Environmental life-cycle of chemicals of emerging concern: new (bio)analytical approaches and environmental risk assessment

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Declaration of authorship

I am the author of this thesis, and the work described therein was carried out by myself personally, with the exception of chapter 2, 3, 4 and 5 where the papers published or being prepared for publishing are the result of contributions from other researchers as well as myself. My contributions are described in the statement of authorship prior to each chapter.

Candidate's signature Kathryn Proctor

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Abstract

Chemicals in the environment are of growing concern as this presence is increasing alongside human population and use. There is increasing evidence that these contaminants of emerging concern (CECs) could drastically impact the environment, despite regulation and legislation in many countries across the world to monitor and limit them.

Current literature concludes there is a need for analytical methods sensitive enough to quantify chemicals from a wide range of classes, e.g. pharmaceuticals, industrial chemicals and pesticides, in various environmental matrices. However, current studies are limited by a small range of chemicals, low method sensitivity and being focussed on aqueous matrices only.

This work captures the development and validation of a highly sensitive ultra-performance liquid chromatography coupled with tandem mass spectrometry method for the analysis of five environmentally relevant matrices. This method has method quantification limits of 0.004 ng L^{-1} (bisphenol A in surface water) to 3,118 ng L^{-1} (creatinine in wastewater influent) and method accuracy and precision of 107.0% and 13.4% for 142 compounds covering 33 different classes. This method was then applied to 5 wastewater treatment works (WwTWs) within the same river catchment in South-West England, providing a detailed high-resolution picture of the levels of chemical contamination and the contribution from the local population.

Within this catchment it was found that raw wastewater entering the WwTWs contained 169.3 kg d^{-1} of CECs, the majority of which was found in the liquid phase, with only 1.4 kg d^{-1} found adsorbed to suspended particulate matter. The population normalised load was very consistent across the catchment with 154 ± 12 mg d^{-1} $inhabitant^{-1}$. Although the WwTWs removed most of the CECs from the liquid phase, there was variation between the WwTWs, with WwTW A only discharging 0.19 kg d^{-1} and 7.3 kg d^{-1} at WwTW E. However, this did correlate highly with the contributing population. Whether the CECs were completely degraded, adsorbed to the solid phase or transformed is unknown, future work should consider the use of untargeted analysis and further sampling of the solid matrices during treatment processes to determine the final fate. Analysis of the solid phases showed a preference for antifungals and antidepressants to adsorb to the matrix. Analysis of the surface water showed many CECs were ubiquitous through the catchment with daily loads ranging from 0.005 g d^{-1} (ketamine) to 1890.3 g d^{-1} (metformin).

This data was then combined with existing ecotoxicity data to provide an environment risk assessment (ERA) of these CECs in this catchment. Overall, it was found that there was minimal risk from individual CECs, however similarly acting chemicals in the same group may pose a much higher risk. It also showed increasing levels of CECs and environmental risk as the river flowed through higher populated urban areas.

A novel ecotoxicity test using the protozoa, *Tetrahymena Thermophila*, was also developed. This test allows smaller quantities of contaminants to be tested but is also highly sensitive and has showed critical evidence of stereospecific ecotoxicity.

Further work should include the use of HR (high-resolution)-MS, analysis of metabolites and transformation products, and the further combination of both chemical and biological approaches.

Chapter 1

Introduction

1.1 The presence of contaminants of emerging concern in the environment

Many chemicals have been detected in the environment around the world; in surface water (Camacho-Muñoz and Kasprzyk-Hordern, 2017; Gogoi et al., 2018; Kümmerer, 2009a; Miller et al., 2017; Noguera-oviedo and Aga, 2016; Rosi-Marshall et al., 2015; Sousa et al., 2018; Wilkinson et al., 2017), groundwater (Lapworth et al., 2012; Stuart et al., 2011), marine water, (B. M. Gustavsson et al., 2017; Minguez et al., 2016), sediments (Díaz-Cruz et al., 2019; Gorga et al., 2015; Kaiser et al., 2012; Kim and Carlson, 2007) and soils (Grossberger et al., 2014; Simpson and McKelvie, 2009; Šudoma et al., 2019; Zhang et al., 2015). They are usually present in the concentration range of ng L^{-1} to µg L^{-1} in the aqueous environment or ng g^{-1} to µg g^{-1} in the terrestrial environment. These contaminants cover a wide range of chemical classes including human and veterinary pharmaceuticals, additives, personal care products, industrial chemicals and pesticides. The entry of these contaminants into the environment is either via point sources, e.g. wastewater treatment works (WwTW) discharges, or diffuse sources, e.g. agricultural application of pesticides. Depending on the physicochemical properties of the chemicals, they may move throughout the environment in-between matrices.

When considering chemicals in the environment, it is important to appreciate the scale and breadth of potential sources. Currently, the European Inventory of Existing Commercial Chemical Substances (EINECs) and the new system, REACH (Restriction, Evaluation, Authorisation and restriction of CHemicals), record a vast number of chemicals (~100,000), very few of which are currently monitored in the environment (ECHA EU, 2009; Loos et al., 2009). The result is a large range of potential CECs, which are further increased in number by the metabolites and transformation products that they may produce. The levels of these CECs in the environment may persist or degrade by a variety of means, including abiotic and biotic processes e.g. photolysis, hydrolysis of microbial metabolism (Andrés-Costa et al., 2017; Vasquez et al., 2014). Priority substances have been identified of high risk to the environment and their use should be minimised. This classification was based upon their widespread presence, persistence, mobility, risk to human health and the environment (Official Journal of the European Union, 2004). A further 11 substances and groups have been prioritised for inclusion in the priority substances list, dependent on further data collection i.e. the Watch List and Water Framework Directive (WFD) ((Carvalho et al., 2015; European Commission, 2015, 2013, 2008, 2006, 2000). These regulatory directives have been initiated to improve and protect environmental water quality, as well as promoting international collaboration. This is essential in Europe, where waterways are not limited by national boundaries.

Overall for many of the chemicals in use, their actual levels, distribution and effects are widely unknown, despite their potential for negative biological effects and hence are being acknowledged as 'chemicals/contaminants of emerging concern' (CECs).

1.2 Monitoring CECs in the environment

The quantification of many of these CECs in the environment is often split between two methods: gas-chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS).

The selection of the method depends on the physicochemical parameters of the CECs. Usually more volatile and/or non-polar CECs are analysed by GC-MS whilst more polar, less volatile compounds are analysed by LC-MS (García-Córcoles et al., 2019; Richardson and Kimura, 2017; Sousa et al., 2018).

In particular, UPLC-MS/MS (ultra-performance liquid chromatography coupled with tandem mass spectrometry) has enabled the widest range of CECs to be analysed at the lowest ranges, suitable for the trace levels found in surface and ground water in the environment, with high accuracy. Furthermore, the use of the soft ionisation method, electrospray ionisation (ESI), coupled with tandem mass spectrometry allows robust compound quantification and confirmation which is particularly important in complex matrices such as wastewater. These methods are now widely used in the monitoring of CECs internationally (Sousa et al., 2018).

Despite this, there is a lot of variation between methods used across different studies, often because of the different equipment in use, the CECs of interest to the study and the matrices to be analysed. This can lead to difficultly in comparing results between studies, due to the use of different methods of sampling, extraction and analysis leading to different levels of accuracy and sensitivity. It requires higher levels of reporting of methods and method validation to begin to draw suitable comparisons (Ort et al., 2010; Ort and Gujer, 2006; Sousa et al., 2018).

Environmental monitoring studies have been limited in scope because they often focus on a specific category of CECs, based on its entry into the environment, e.g. pharmaceuticals from households or hospitals (Emmanuel et al., 2005; Paíga et al., 2019), veterinary medicines or pesticides from aquaculture or agriculture (Ccanccapa et al., 2016; M. Gustavsson et al., 2017; Masiá et al., 2015), industrial chemicals or personal care products ingredients from household, trade or production effluents (Hussain et al., 2018; Petre et al., 2014). Many studies focus on specific classes often based upon similar uses or their mode of action, such as antibiotics (Baquero et al., 2008; Gros et al., 2013), antidepressants (Ribeiro et al., 2014), anticancer drugs (Negreira et al., 2013), illicit drugs (Pal et al., 2013), pesticides (Ccanccapa et al., 2016; M. Gustavsson et al., 2017; Masiá et al., 2015) and endocrine disrupters (Chang et al., 2009). CECs of high interest or high risk have also been a focus of study, e.g. priority substances and persistent organic pollutants (Estevez et al., 2016; Reemtsma et al., 2016). There is also the risk of the 'Matthew Effect', where further monitoring is biased towards CECs which have already been

detected over those which have not yet been considered (Daughton, 2014), something which the NORMAN Network aims to prevent, amongst other objectives (Network of reference laboratories, research centres and related organisations for the monitoring of emerging environmental substances, (Dulio et al., 2018)).

Much of current literature and research has focused primarily on aqueous matrices, as these are both simpler to analyse and between aqueous and solids matrices, have the potential for greater transport through the environment. Organisms have higher levels of contact with aqueous matrices and are therefore more likely to be at more risk of adverse effects. Additionally, solid matrices are often not considered due to complex matrix effects and time-consuming sample preparation (Petrie et al., 2014).

Studies focusing on aqueous matrices have considered a variety of different aspects (Gogoi et al., 2018; Luo et al., 2014). Influent is often a focus for wastewater based epidemiology, to not only understand human behaviour through their licit and illicit drug use (Castrignanò et al., 2016; Mastroianni et al., 2017) but also in the context of public health monitoring (Choi et al., 2018; Rice and Kasprzyk-Hordern, 2019). This is due to the ease of which a sample, representative of the entire population, can be collected. Previously data on drug use or tracking epidemics or pandemics were done through surveys, individual urine sample collections or a rise in cases at hospitals or GP surgeries. However, by analysing the wastewater of a population for chemicals, metabolites and biomarkers of interest this data can potentially be provided much more accurately and with minimal bias. This method of gathering data on the health of a population would be much closer to real-time and additionally would provide information on spatial and temporal trends.

Considering both influent wastewater (untreated) and effluent wastewater (treated) can provide evidence to understand effective treatment technologies for the removal of CECs (Wang and Wang, 2016), factors which effect removal (Kumar et al., 2019; Tsui et al., 2014) and minimising entry of potential high risk chemicals into the environment (Gros et al., 2007; Kasprzyk-Hordern et al., 2009; Martín et al., 2012). Furthermore, monitoring effluent allows quantification of chemicals entering the environment prior to dilution, allowing the calculation of predicted environmental concentrations. This can be useful in cases where the environmental concentrations are too low to be monitored but may still pose a risk.

A better understanding of the risk posed by wastewater effluent to the environment can come from comparing the environmental concentrations upstream and downstream of the wastewater effluent discharge point (Acuña et al., 2015). This can quantify the environmental burden of the wastewater treatment plant and local population, and provide a more accurate environmental risk assessment of the CECs of interest (Riva et al., 2019).

Groundwater is also often analysed. This is water that has been 'filtered' by the surrounding soil etc. and can further understanding of the mobility of some compounds, particularly those which are not adsorbed onto soil, and may pose the most risk to drinking water abstraction sources (Lapworth et al., 2012; Postigo and Barceló, 2015; Stuart et al., 2012).

Drinking water itself is also monitored as some CECs have been found in drinking water sources and may not be adequately removed during drinking water treatment (Metcalfe et al., 2014). Furthermore, transformation products of these compounds are of particular interest, as these compounds may persist through later stages of the water treatment process and are a potential risk to humans (Huang et al., 2020; Postigo and Richardson, 2014).

1.3 The biological effects of CECs

The presence of CECs in the environment can cause significant adverse effects to the ecological balance. Consider the food web, which consists of a wide range of different organisms, spanning several trophic levels. Viewed as a pyramid, the lower trophic levels such as algae and microorganisms are many in number and support the higher trophic levels. Drastic changes to one layer of the food web may cause other layers to grow out of control or collapse, particularly if keystone species are affected (Clements et al., 2012; Clements and Rohr, 2009; Grove et al., 2009; Rohr et al., 2006). This is a delicate balance and often human interference with the environment has caused drastic effects, e.g. overfishing or deforestation. Although trace level chemical pollution may not result in more subtle outcomes than these examples, it can still have a serious impact on the long-term future of organism populations e.g. feminisation of fish (steroidal estrogens and xenoestrogens) (Jobling et al., 2009, 1998; Kidd et al., 2007), drastic reduction in the population of vultures in Pakistan (diclofenac) (Oaks et al., 2004), behavioural changes in fish (sertraline) (Hedgespeth et al., 2013), amphipods and starlings (fluoxetine) (Bean et al., 2014; Kohler et al., 2018) and increasing levels of antibiotic, antifungal, pesticide and herbicide resistance(Hawkins et al., 2019; Kümmerer, 2009b, 2004; Peterson et al., 2018; Schütte et al., 2017). The results from these investigations have led to a more proactive approach to environmental risk assessment, particularly with the growing understanding of the potential impact of sublethal and chronic effects to the environment, rather than just acute toxic effects and the potential for bioaccumulation and bioconcentration (Katagi, 2010; Zenker et al., 2014).

1.4 Understanding complexity and quantifying the (potential) effects of CECs in the environment

Understanding and quantifying the effects of CECs in the environment is a complex process as different concentrations and exposure characteristics of CECs can produce varying effects in organisms covering several trophic levels. Furthermore, the presence of other substances, other sources of stress or different experimental factors may influence these effects (Boström and Berglund, 2014; Noguera-oviedo and Aga, 2016).

To investigate this complex situation, studies often simplify the study to within a lab setting by focusing on one condition at a time, i.e. a single compound and varying concentration, exposure time, organism etc. Many toxicity studies have previously only focused on acute effects (end points) i.e. immobility and death, from high concentrations over short periods (Santos et al., 2010). However, it is becoming increasingly understood that the effects of an organism's longer term exposure to lower concentrations is far more relevant to the environment. Therefore, there is now an increasing focus on chronic ecotoxicity studies, which are carried out with lower concentrations over a longer period, i.e. several days to weeks, to understand the effects of exposure over several generational cycles of the organism (long-term effects) (European Medicines Agency, 2018; Santos et al., 2010). The end point may still include mortality or immobility, but more often focus on fecundity, size, and behaviour. These tests usually cover several concentrations to form a dose-response curve, from which the EC_{50} is determined (effect concentration at which 50% of the population is affected, similar to LC_{50} – lethal concentration at 50% of the population has died) (Backhaus and Faust, 2012; European Medicines Agency, 2018; Laws, 2013). The EC_{10} (concentration at which 10% of the population is affected), the LOEC (lowest observed effect concentrations) and the NOEC (no-observed effect concentration) can also be calculated from these curves.

There are limitations to the ecotoxicity data currently available:

- EC_{50} and LC_{50} data are still far more widely available than EC_{10} or LOECs for the majority of compounds across a variety of organisms. Therefore, studies which investigate more novel chemicals need to carry out ecotoxicity tests to fill the gaps in the data or use predictive models such as 'Quantitative structure activity relationship' (QSAR).

- Different end points can result in different effect concentrations ($EC_{10,}$ EC_{50}), as they will be dependent upon the mode of action of the chemical. Furthermore, not all end points have been investigated for all compounds in all organisms.

- A large proportion of the currently available ecotoxicity data is primarily available for aquatic organisms and there is limited information available for the terrestrial compartment.

- Focus on individual chemicals - The ecotoxicity values are based on an individual chemical. The exposure data from the numerous studies of CECs in the environment suggests that organisms are rarely exposed to a single chemical. Therefore, these

laboratory-based toxicity tests do not provide an accurate representation of the environmental effect since it does not consider the potential synergistic or antagonistic effects of other CECs, or even the simplest effect of concentration addition of similarly acting compounds. A single compound itself can become a mixture in the environment, as it may be metabolised by organisms or transformed by the processes of hydrolysis or photolysis.

1.5 Environmental Risk Assessment

Despite environmental monitoring showing that CECs are ubiquitous in the environment, the potential effects remain difficult to quantify, identify or predict. Currently the risk of individual chemicals to the environment is assessed at the regulative level e.g. European Medical Agency (EMEA), or REACH (European Commission, 2006; European Medicines Agency (EMEA), 2006; European Medicines Agency, 2018). These environmental risk assessments (ERAs) consider the main active ingredient in each formulation and potential additives of metabolites that are >10% of the main active ingredient. From this they calculate the potential usage of these products and predict the environmental concentrations (PECs, predicted environmental concentrations). There are many limitations to this approach whilst predicting exposure levels (PECs); primarily the number of assumptions that must be made regarding, usage, human metabolism (in the case of pharmaceuticals), WwTW treatment efficiency, dilution to the environment, to name a few. These PECs are then compared with predicted no-effect concentrations (PNECs) to provide a risk quotient (RQ). An RQ greater than 1 shows that there may be a risk to the environment and more work is required to increase the accuracy of the prediction.

In the assessment of environmental risk, the EC_{50} or LC_{50} is used as an acute ecotoxicity parameter and lower levels such as the EC_{10}, LOEC or NOEC are typically used as chronic ecotoxicity parameters (European Medicines Agency, 2018). For ERAs, traditionally EC_{50}s were used in the calculation of predicted-no-effect-concentrations (PNEC) along with an assessment factor (AF) (Backhaus 2012, 2014). However, there is a growing use of EC_{10} in these calculations as it requires a lower AF since it is more representative of the environment. Therefore, recent guidelines advise the use of chronic ecotoxicity data, with a preference for the use of EC_{10} as these are more likely to be statistically determined, rather than the LOEC and NOEC, which are often not statistically determined (European Medicines Agency, 2018). Also, since different organisms can have varying responses to specific chemicals, it is standard to compare the ecotoxicity results to three organisms from different trophic levels within an environmental compartment e.g. freshwater aquatic systems, marine aquatic systems, sediments, and soils (European Medicines Agency, 2018), to ensure the more sensitive trophic levels are considered and provide a more complete picture to the total environment. However, there is limited chronic toxicity data available for all but the freshwater aquatic systems.

The ecotoxic concentrations which are obtained from these studies are converted to a PNEC based on the lowest EC_{50} or EC_{10} or LOEC if available and divided by an assessment factor (AF). AF are included in the ERA as a deliberate safety margin to account for limitations in the estimations of risk such as the use of lab-raised organisms in ecotoxicity and the lack of inter- and intra-species variation in sensitivity, which will limit how representative the tests are to the environment and real-life situations. This may also provide more time to mitigate the risk to the environment by preventing the use of or implementing treatment technologies in the case of the PEC reaching the PNEC, resulting in a RQ >1, which would show the potential risk to the environmental is high (European Medicines Agency, 2018).

As previously stated, the environment contains a complex mixture of CECs and therefore the potential effect of the mixture should be considered as well as the individual components, though this is not carried out at a regulative and legislative level. There are two models which can be used in considering the effects of mixtures. The first, concentration addition (CA), assumes that all components contribute proportionally, based on their individual PEC/PNEC, to the overall effect on a single target system. The second model, independent action, (IA) assumes that all components contribute to the overall effect, but via different modes of action. Although IA describes a situation that more clearly resembles a mixture in the environment, it does not consider potential synergistic or antagonistic actions of the mixtures' constituents and therefore in practice, the ERA of the mixture often falls between IA and CA. For this reason, CA is often applied in ERA as it provides a slightly more cautious risk assessment (Backhaus and Faust, 2012; European Medicines Agency, 2018).

A further limitation of ERAs is the lack of consideration of stereochemistry. It is well known in the pharmaceutical industry that differences in stereochemistry in pharmaceuticals have different levels of potency in humans, e.g. S-(-)-ibuprofen is 100 times more potent than R-(+)-ibuprofen (Buser et al., 1999), and may lead to different biological effects e.g. R-(+)-thalidomide acts as a sedative and S-(-)-thalidomide is teratogenic (Höglund et al., 1998; Wnendt et al., 1996). Enantiomers are even known to have the potential to cause antagonistic effects towards their antipode e.g. R-(-)-citalopram inhibits S-(+)-citalopram (Sánchez et al., 2004), (Evans and Kasprzyk-Hordern, 2014). Yet despite these factors, the effects of varying enantiomeric fractions (EFs) on non-target organisms is only known for a few compounds (Petrie et al., 2014).

Aims and Objectives

Overall, this work will implement novel analytical and bioanalytical methods to investigate the fate and effects of CECs in the environment. Chemical and biological methods need to be combined to provide evidence on both exposure and effects of CECs in the environment. This is with the aim to provide higher levels of accuracy with regards to the use of PECs in ERAs by not only replacing them with

measured environmental concentrations (MECs) but also with consideration of spatial and temporal variations to provide a more robust ERA.

Both solid and liquid matrices will be sampled and analysed to provide a more holistic understanding of the levels of exposure to CECs that are experienced by organisms in different environmental compartments. To achieve this, CECs will need to be prioritised for development of a targeted multi-residue method using UPLC-MS/MS, for the quantification of CECs in both solid and liquid environmental matrices at trace level. Furthermore, the impact of WwTWs on the presence of CECs will be considered and evaluated, based on spatial and temporal trends of the CECs entering the WwTWs and being discharged into the environment via effluent and digested solids.

Effect driven approaches will then be considered, using current ERA techniques to assess the environmental risk of the CECs, both individually and as a mixture, for both the aquatic and terrestrial environments. For the aquatic environment, this will be investigated at a local and catchment level, to determine the impact of WwTW effluent to the environment. Furthermore, this work will consider gaps and limitations of current ERA approaches and develop new tools and recommendations for improvements to ERAs.

Objectives:

1) The first objective will be to develop and validate a new multi-residue UPLC-MS/MS method which contains a wider range of CECs than previously published, for both solid and liquid matrices (Chapter 2).

2) The next objective will be to apply the newly validated method in a catchment-based study to several key matrices, wastewater influent (both solid and liquid phases), treated effluent, digested solids and the receiving surface waters to which the effluent is discharged. This will further understanding on the removal of CECs within WwTWs and entry into the environment. This will also determine levels of exposure experienced by aquatic and terrestrial organisms (Chapter 3).

3) The measured environmental concentrations will be used to assess the environmental risk of these levels of exposure within the catchment to aquatic and terrestrial organisms, for both individual CECs and the overall mixture (Chapter 4). This will provide a more robust and ERA for a wide range of CECs within this catchment.

4) Several limitations have been identified in literature regarding ecotoxicity tests and therefore this area will be explored more in-depth with the aim to develop novel approaches and further ecotoxicological considerations (Chapter 5).

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Appendix 6B: Statement of Authorship

This declaration concerns the article entitled:									
Multi-residue ultra-performance liquid chromatography coupled with tandem mass spectrometry method for comprehensive multi-class anthropogenic compounds of emerging concern analysis in a catchment-based exposure-driven study									
Publication status (tick one)									
Draft manuscript		**Submitted**		**In review**		**Accepted**		**Published**	X
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Copyright status (tick the appropriate statement)									
I hold the copyright for this material		Copyright is retained by the publisher, but I have been given permission to replicate the material here	X						
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	Formulation of ideas: The candidate was a major contributor on the prioritisation of CECs for the work and interpretation of data. Design of methodology: The candidate was a minor contributor as the methods used were to provide comparable data to previously published work Experimental work: The candidate was a major contributor in the collection of samples, sample preparation and analysis for all novel CECs and previously unpublished work. Presentation of data in journal format: The candidate was a major contributor in the writing of the paper and the ideas expressed.								
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.								
Signed	[redacted]					**Date**	11/12/2020		

Published Paper

Multi-residue ultra-performance liquid chromatography coupled with tandem mass spectrometry method for comprehensive multi-class anthropogenic compounds of emerging concern analysis in a catchment-based exposure-driven study

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PAPER IN FOREFRONT

Multi-residue ultra-performance liquid chromatography coupled with tandem mass spectrometry method for comprehensive multi-class anthropogenic compounds of emerging concern analysis in a catchment-based exposure-driven study

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Abstract

This paper presents a new multi-residue method for the quantification of more than 142 anthropogenic compounds of emerging concern (CECs) in various environmental matrices. These CECs are from a wide range of major classes including pharmaceuticals, household, industrial and agricultural. This method utilises ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) for analysis of five matrices (three liquid and two solid) from wastewater treatment processes and the surrounding environment. Relative recoveries were predominantly between 80 and 120%; however, due to the complexity of the matrices used in this work, not all compounds were recovered in all matrices, from 138/142 analytes in surface water to 96/142 analytes in digested solids. Method quantification limits (MQLs) ranged from 0.004 ng L^{-1} (bisoprolol in surface water) to 3118 ng L^{-1} (creatinine in wastewater treatment work (WwTW) influent). The overall method accuracy was 107.0%, and precision was 13.4%. To test its performance, the method was applied to the range of environmental matrices at WwTWs in South West England. Overall, this method was found to be suitable for application in catchment-based exposure-driven studies, as, of the total number of analytes quantifiable in each matrix, 61% on average was found to be above their corresponding MQL. The results confirm the need for analysing both the liquid and solid compartments within a WwTW to prevent under-reporting of concentrations.

Keywords Chemicals of emerging concern · Analysis · Environment · Mass spectrometry

Introduction

The use of anthropogenic, household, industrial or agricultural chemicals such as pharmaceuticals, pesticides, plasticisers, UV filters, industrial chemicals and microplastics is ubiquitous, and they have been recognised as a source of environmental contamination. These compounds have been quantified at levels ranging from ng L^{-1} to μg L^{-1}, and their impact on the environment is not well known. These compounds are often designed to be biologically active and can be persistent in the environment, where they have the potential to bioaccumulate within the tissues of organisms [1–4]. For these reasons, among many others, they are known as compounds of emerging concern (CECs) [5–7].

CECs are primarily introduced to the environment via point sources such as wastewater treatment works (WwTWs), industrial discharge points and landfill leachates [8–11]. Diffuse sources, such as direct application to land in agriculture, have

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been identified as a source of select sub-classes of CECs, such as pesticides and veterinary pharmaceuticals. Additionally, the application of digested sludge, from WwTW processes, directly to the land in modern farming practices is a potential source of other classes of CECs [12]. Bisphenol A (BPA) for instance, has been found at high levels within this matrix [13, 14].

CECs have been detected across the world in a multitude of environmental matrices [6, 15, 16]. This is due to their widespread use and to partitioning that can occur from the aqueous phases into suspended solids and sediments, where it can affect terrestrial organisms and fauna. Whilst their presence does not necessarily mean harm, the ecotoxicological effects of many of the CECs have been quantified in laboratory-based studies for a variety of different organisms across trophic levels and toxic effects have been demonstrated [17–19]. These laboratory-based ecotoxicological studies broadly focus on a single compound versus a single organism, but the environment is a 'cocktail' of CECs and different microorganisms. Ermler et al. [20] addressed this lack of knowledge for anti-androgens and found that concentration addition is a good model for predicting the effect of mixtures for up to six compounds. Leading on from this work, Orton et al. [21] tested multi-component mixtures of up to 30 compounds with varying mixture ratios. At the point where one mixture caused a 10% inhibition of the cancer cell assay, the concentrations of the components were a factor of 5.8 lower than the concentration that would be needed for them to individually cause this effect [21]. This highlights the need for current analytical methods to have method quantification limits (MQLs) lower than the no-observed effect concentrations (NOECs) of individual CECs, to enable accurate measurements of very low concentrations, for a better understanding of the risk they pose to the environment in combination.

Work is being done to further understand the fate, behaviour and effect of CECs within our environment. However, the sheer number of them and their everchanging usage makes this a challenge. The European Inventory of Existing Commercial Chemical Substances (EINECS) contains over 100,000 substances [22], with further substances being registered across all EEA countries, through Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Currently, there are 94,705 registrations containing 22,257 substances, or 22,096 unique substances that are in use [23]. This number is increasing every year, as new substances are developed and registered, and this pattern can be found across all classes of CECs.

The exponential growth of populations across the world, due to increased life expectancy and decreased infant mortality, is increasing the usage of pharmaceuticals. This puts further pressure on agriculture to produce food faster and cheaper, often via the use of pesticides, herbicides and other anthropogenic compounds. With the number of CECs in use, and more being developed each year, it is not feasible to determine the exposure and effects of all these compounds in a single catchment, let alone across a country or continent. To further complicate matters, many CECs degrade through different processes such as hydrolysis, photolysis and metabolism. These form transformation products and metabolites, which are often more harmful than the parent compounds [24].

Brack et al. [25] conducted a review of the EU Water Framework Directive (WFD) and concluded that there are specific challenges both at European scale and at a local scale. Regulation and national monitoring schemes such as the WFD and the UK Chemical Investigations Programme (CIP) are suitable for furthering the understanding of this problem on a wider scale. Through identification and assessment of the most widespread CECs, high-risk compounds can be identified and managed through harmonised methods across diverse areas. However, they are often limited by the sampling method, i.e. a few samples collected at many locations, using only grab samples, which are not very representative [26]. Sampling at a local scale is crucial to determine catchment-specific substances and mixtures that might be a specific problem in the local environment. This can allow more targeted management of risks and hazards at local catchment level. There is a further need for analytical methods which can detect CECs down to, or below, the predicted no-effect concentration (PNEC), as this will allow a more adequate risk assessment [27].

To gain a better understanding of the fate of CECs within an environmental catchment, analysis of the influent and effluent of contributing WwTWs is required, including analysis of solid particulate matter (SPM) and digested solids, as well as corresponding surface water. Overall, in the literature, there is still a lack of multi-residue methods for quantification of CECs in solid matrices. Even fewer publications consider both the liquid and solid phases in the WwTW or the environment. Many studies focus specifically on pharmaceuticals, personal care products, industrial chemicals, veterinary pharmaceuticals or pesticides. However, a catchment potentially contains compounds from a variety, if not all, classes of CECs, though it is rare that they are all the focus of analysis in a single campaign. One study by Gustavsson et al. [28] covers many different classes of CEC, leading to the analysis of 172 compounds; however, this was only achieved through multiple sample preparation methods. For a comprehensive understanding of the exposure and environmental risk of chemical mixtures, multi-residue quantitative methods covering a large variety of CECs are required.

The aim of this work is to develop and validate a new multi-residue method (< 190 compounds including internal standards) for a wide range of CECs prioritised for risk assessment at a catchment scale, and accounting for the highly urbanised and agricultural areas of one catchment. The classes of CECs covered by this method include the following: UV filters, parabens, plasticisers, steroid estrogens, antibacterials/antibiotics, antifungals, hypertension drugs, non-steroidal anti-inflammatory drugs

(NSAIDs), lipid regulators, antihyperlipidaemics, antihypertensives, antihistamines, drugs for erectile dysfunction, drugs for diabetes, cough suppressants, beta blockers, H_2 receptor agonists, X-ray contrast media, drug precursors, anticancer drugs, anaesthetics, antidepressants, anti-epileptics, calcium channel blockers, hypnotics, antipsychotics, drugs for dementia, human indicators, analgesics, stimulants, opioids, drugs used in veterinary medicine, pesticides, fungicides and herbicides and metabolites. The selection of analyte groups was based not only on prioritisation including existing and proposed legislation, European and national watch lists (UKWIR CIP, EU Watch List) [29–32] and the literature [33, 34] but also on exploring usage statistics (NHS prescriptions [35]), entry into the environment (metabolism, excretion from DrugBank [36]), persistence, bioaccumulation, transport throughout the environment and toxicity of organisms [mammals, aquatic and benthic (log K_{ow}, log K_{oc}, log D_{ow}, water solubility, vapour pressure, Henry's law constant, bioconcentration factor, EPI Suite, ACD/Labs [37, 38])].

Materials and methods

This paper provides an expanded and broader scope method based on the method published by Petrie et al. [39]. Electronic Supplementary Material (ESM) Table S1 contains data on the suppliers of the compounds as well as their physiochemical properties.

The analytes were primarily purchased in solid form, before being accurately weighed and dissolved in HPLC grade methanol (MeOH) (Sigma-Aldrich), or other suitable solvents, at a concentration of 1.0 mg mL^{-1}. These stock solutions were stored in silanised glass vials in the dark at − 20 °C, unless otherwise stated. Multi-analyte mixtures were prepared from these stock solutions. The CECs, their corresponding internal standards, data acquisition method and MS/MS detection parameters can be found in ESM Table S2. Chromatograms of all analytes can be found in ESM Fig. S1.

The internal standards 1*S*,2*R*-(+) ephedrine-d3, amphetamine-d5, benzoylecgonine-d8, cocaethylene-d8, cocaine-d3, codeine-d6, cotinine-d3, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrroloidine-d3 (EDDP-d3), estradiol (2,4,16,16-d4), estrone (2,4,16,16-d4), heroin-d9, ketamine-d4, 3,4-methylenedioxyamphetamine-d5 (MDA-d5), 3,4-methylenedioxymethamphetamine (MDMA-d5), mephedrone-d3, methadone-d9, methamphetamine-d5, methylparaben-13C, morphine-d3, norketamine-d4, quetiapine-d8 and tempazepam-d5 were purchased from LGC Standards (Middlesex, UK). Amitrpityline-d3, amoxicillin-d4, capecitabine-d11, ciprofloxacin-d8, citalopram-d6, diazepam-d5, erythromycin-13C,d3, fluoxetine-d5, gabapentin-d4, imidacloprid-d4, metazachlor-d6, metoprolol-d7, mirtazapine-d3, norsertraline-d4, nortriptyline-d3, ofloxacin-d3, oxazepam-d5, sildenafil-d8 and verapamil-d7 were purchased from Toronto Research Chemicals (TRC) (Toronto, Canada). Acetaminophen-d4, atenolol-d5, bisphenol A-d16, carbamazepine-13C6, ibuprofen-d3, ketoprofen-d3, metformin (dimethyl-d6), methiocarb-d3, naproxen-d3, propranolol-d7, sertraline-d3 and tamoxifen-13C2,15N were purchased from Sigma-Aldrich (Gillingham, UK), and bezafibrate-d6 was purchased from QMX Laboratories (Thaxted, UK). These were purchased as solutions at a concentration of 0.1 mg mL^{-1} or 1.0 mg mL^{-1} in methanol or other appropriate solvents. If no solutions were available, 1.0 mg powder was purchased, and the entire contents of the vial were dissolved in methanol. The MS/MS detection parameters for the internal standards can be found in ESM Table S3.

All glassware in this paper was silanised to prevent the analytes and internal standards from absorbing to the surface. This was done by coating the internal surfaces of the glassware with 5% dimethylchlorosilane (DMDCS) in toluene (Sigma-Aldrich), rinsing with toluene (Sigma-Aldrich) twice, then rinsing again with MeOH three times, and leaving to dry between each coating or rinse.

Methods for sample collection

Sampling was carried out in a river catchment in South West England. Sampling involved collection of samples from all major WwTWs and receiving environmental waters. Twenty-four-hour composites using a 3700 ISCO sampler (RS Hydro) were collected for both influent and effluent wastewaters in each case. Grab samples were utilised in surface water samples. SPM was collected from influent samples.

SPM per litre was calculated by filtering 30 mL through a pre-dried and pre-weighed GF/F glass microfibre filter. This was then re-dried at 105 °C for 8 h, left to cool and re-weighed to quantify SPM in grams per litre. Data are shown in ESM Table S4. Digested sludge was collected on three consecutive days, both directly after digestion and prior to disposal. Data are shown in ESM Table S5.

All samples were kept on ice during sampling (ice was placed within the composite sampler to maintain a cool temperature of 0–4 °C and promote stability) or placed in a cool box and kept on ice until the samples were transported to the lab. Once at the lab, samples for liquid analysis were transferred to 125-mL PPE bottles (Fisherbrand) and frozen (− 20 °C) for further preparation and analysis at a later date. For the influent samples, the remainder of the sample was filtered to collect the SPM, which was then frozen (− 20 °C). Most compounds do not adsorb to the PPE bottles, with very few exceptions (ESM Table S6).

Methods for extraction and analysis

The methods used for sample preparation of both liquid and solid matrices, as well as their analysis, can be found

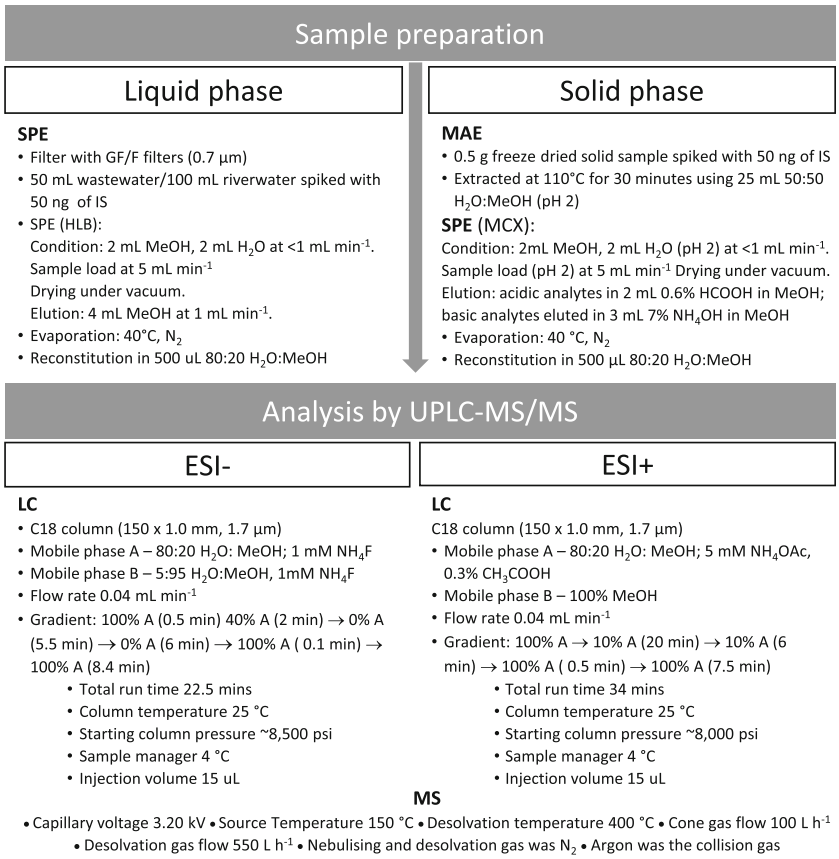

Fig. 1 Flow chart from sample preparation to analysis, for analysis of liquid and solid samples by ESI− and ESI+ methods

in Fig. 1; this is also discussed in more detail below. Development of an extraction method for liquid matrices with hydrophilic-lipophilic-balanced solid-phase extraction (HLB SPE) was developed based on the method published by Kasprzyk-Hordern et al. [40]. The microwave-assisted extraction (MAE) for solid matrices was developed based on the method published by Petrie et al. [39].

Extraction for liquid matrices

The samples were filtered with a GF/F glass fibre filter (0.7 µm) (Whatman, Sigma-Aldrich) and adjusted to pH 7.5–8.5. 50 mL of influent or effluent (100 mL surface water) was accurately measured and spiked with 50 µL of 1 µg mL^{-1} (50 ng) internal standard mixture in MeOH. The 60-mg HLB SPE cartridges (OASIS, Waters, UK) were conditioned and equilibrated with 2 mL of MeOH, followed by 2 mL of deionised water (H_2O) at a rate of < 1 mL min^{-1} (under gravity). The deionised H_2O was obtained from a Milli-Q system (18.2 MΩ). The sample was loaded at 5 mL min^{-1} before the cartridges were dried under vacuum. Analytes were eluted using 4 mL MeOH at 1 mL min^{-1} (under gravity). Once eluted, the extracts were evaporated to dryness at 40 °C, with a steady flow of nitrogen using a TurboVap LV concentration workstation. Finally, the samples were reconstituted in 500 µL of 80:20 H_2O:MeOH, mixed thoroughly to ensure completed dissolution and transferred to LC vials (polypropylene) (Waters, UK).

Extraction for solid matrices

The solid samples, digested sludge and SPM were initially frozen and freeze-dried (ScanVac, CoolSafe freeze dryer, Lynge, Denmark). The freeze-dried samples were homogenised, and 0.25–0.5 g was weighed out and spiked with 50 µL of 1 µg mL^{-1} (50 ng) internal standard mixture in MeOH. This was left for 30 min to 1 h, for the methanol to evaporate off. The samples were then transferred to MAE perfluoroalkoxy (PFA) tubes with 25–30 mL of 50:50 acidified H_2O (pH 2):MeOH. The MAE tubes were placed in 800 W MARS 6 microwave (CEM, UK). The temperature was ramped to 110 °C, over 10 min, then held at this temperature for 30 min, before allowing the samples to cool. SPE was then carried out using 60-mg mixed-mode cationic exchange (MCX) cartridges (Oasis, Water, UK). These were conditioned and equilibrated using 2 mL MeOH and 2 mL acidified H_2O (pH 2) at < 1 mL min^{-1} under gravity. The samples were then loaded at 5 mL min^{-1} and dried under vacuum. Once dried, the acid analytes were eluted first with 2 mL of 0.6% formic acid (HCOOH) (Sigma-Aldrich) in MeOH. The basic analytes were eluted second with 3 mL of 7% ammonium hydroxide (NH_4OH)

in MeOH (Sigma-Aldrich). These extracts were evaporated to dryness at 40 °C, with a steady flow of nitrogen using a TurboVap. The residue was reconstituted in 500 μL of 80:20 H_2O:MeOH, mixed thoroughly to ensure completed dissolution and transferred to LC vials.

Analysis of samples

The analytes were separated by UPLC performed on the Waters ACQUITY UPLC™ system (Waters, UK). The column used was a reversed-phase C18 column (Waters, UK), 150 mm × 1.0 mm, with a particle size of 1.7 μm. The samples were analysed with Xevo Triple Quadrupole (TQD) Mass Spectrometer (Waters, UK), equipped with an electrospray ionisation (ESI) source in positive and negative modes. To optimise ionisation, two different sets of parameters were used for the ESI-positive and ESI-negative modes. The parameters for these can be seen in Fig. 1. The systems were controlled using MassLynx (Waters, UK). Argon (99.998%) gas, supplied by a BOC cylinder, was used as a collision gas. The nebulising gas was nitrogen, provided by a high-purity nitrogen generator (Waters, Manchester, UK). Two mobile phases were used in the gradient mode ESI+: mobile phase A contained 80:20 H_2O:MeOH with 5 mM ammonium acetate (NH_4OAc) and 3 mM acetic acid (CH_3COOH) (pH 4.7), and mobile phase B contained 100% MeOH. Starting conditions were 100% A, decreasing to 10% A over 20 min, maintained at this level for 6 min, before increasing back to 100% A over 0.5 min and held for 7.5 min to return the column to equilibrium; in ESI−: mobile phase A contained 80:20 H_2O:MeOH with 1 mM ammonium fluoride (NH_4F) and mobile phase B, which was 5:95 H_2O:MeOH containing 1 mM NH_4F. The gradient began at 100% A for 0.5 min and reduced to 40% A over 2 min, before being further reduced to 0% A over the next 5.5 min. It was held at 0% A for 6 min before increasing back up to 100% A over 0.1 min. This was maintained for 8.4 min to re-equilibrate the column. The HPLC grade MeOH, NH_4OAc (Fluka) and CH_3COOH were obtained from Sigma-Aldrich (Gillingham, UK). The NH_4F (Fluka) was obtained from Fisher Scientific (Loughborough, UK) and deionised water (18.2 MΩ) obtained from a Milli-Q system. The temperature of the built-in sample manager was 4.0 °C with an injection volume of 20 μL. The mobile phases were run at a rate of 0.04 mL min^{-1}, as a gradient of a high ratio of aqueous (80%) to 100% MeOH in both positive and negative ionisation modes. The exact gradients and composition of the mobile phases can be also seen in Fig. 1. Data processing was carried out using TargetLynx software, which is an extension to MassLynx (version 4.1, Waters).

Instrument performance

To quantify the analytes, an internal standard approach with an 18-point calibration curve was used. For the majority of the compounds, the analysed range covers 6 orders of magnitude, from ng L^{-1} to mg L^{-1}. Each point was repeated every 24 h over 3 days. These calibration samples were prepared in a ratio of 80:20 H_2O:MeOH unbuffered solutions. The signal-to-noise ratios of these samples were used to determine the concentration of the instrument detection limit (IDL) and instrument quantification limit (IQL), where S/N ≥ 3 or S/N ≥ 10, respectively. Determination coefficients (r^2) were calculated for the full linear range (IDL to ≤ 1000 μg L^{-1}). Inter- and intra-day precision and accuracy were calculated from repeated injections, at regular intervals ($n = 3$) of three concentrations (10 μg L^{-1}, 100 μg L^{-1}, 500 μg L^{-1}) in 80:20 H_2O:MeOH, across 24 h (intra-day) and across 72 h (inter-day).

Method performance

As SPE was used as a preconcentration and clean-up step, the recovery of each analyte must be assessed. Absolute and relative recoveries for SPE of liquid matrices were calculated from matrices spiked in duplicate ($n = 2$) at three concentrations for A-ESI+ (100 ng L^{-1}, 1000 ng L^{-1} and 5000 ng L^{-1} for effluent and influent, and 50 ng L^{-1}, 500 ng L^{-1} and 2500 ng L^{-1} for surface water). For B-ESI+ and C-ESI−, the matrices were spiked at two concentrations (100 ng L^{-1} and 1000 ng L^{-1} for effluent and influent, and 50 ng L^{-1} and 500 ng L^{-1} for surface water). For influent suspended particulate matter (SPM) and digested sludge (DS), the absolute and relative recoveries take into account MAE and SPE. The samples were spiked at 50 ng g^{-1} and 100 ng g^{-1}.

Method detection limits (MDLs) and MQLs were calculated using Eq. 1

$$\mathrm{MDL} = \frac{\mathrm{IDL} \times 100}{\mathrm{Rec} \times \mathrm{Cf}} \quad (1)$$

where IDL is the instrumental detection limit, which is calculated as discussed in the section "Method performance"; 100 is the conversion factor for recovery of a specific matrix (Rec); and Cf is the concentration factor for the specific liquid matrix, e.g. 200 for surface waters or 100 for effluent of effluent. For solid matrices, Cf is replaced with a conversion factor of 2, which converts the volume into grams, based on the 0.25 g of solid matrix being extracted into a 0.5-mL vial for analysis. MQL is calculated with the same equation but by replacing IDL with IQL.

Furthermore, the accuracy and precision of the overall method, including SPE, are also required. These were calculated from samples of 3 matrices taken from 5 different WwTWs in the South West UK. These samples were spiked at 100 ng L^{-1} and 1000 ng L^{-1} for influent and effluent samples and at 50 ng L^{-1} and 500 ng L^{-1} for surface water for A-ESI+ and at 100 ng L^{-1} for all matrices for B-ESI+ and C-ESI−. The accuracy of the method was determined from the

percentage deviation from the known concentration of analyte added to the sample. Precision was calculated as the relative standard deviation (RSD) of the replicates.

It has been found that complex matrices such as influent can affect the detection of analytes, especially when these samples have undergone SPE with HLB cartridges, as these cartridges extract a huge range of compounds. Therefore, matrix suppression was determined for the liquid matrices, which were extracted by employing a method using this approach. Samples for calculating matrix suppression were prepared by spiking samples with 50 μL of 1 μg mL^{-1} of internal standards, after the elution step of SPE, and prior to evaporation and reconstitution. Once analysed, matrix suppression for each analyte was calculated using the following equation:

$$\text{Matrix suppression} = 1-\left(\frac{\text{PA in SS}-\text{PA in US}}{\text{PA in MP QC}}\right) \quad (2)$$

where PA is the peak area of the analyte in spiked sample (SS), unspiked sample (US) and mobile phase quality control (MP QC) sample.

All matrices used were collected via grab sampling and homogenised, and all analyses were carried out on this single sample to ensure consistent results. Due to the use of these environmental matrices and the potential presence of analytes within the matrices prior to spiking, the 'blank' or unspiked (with analytes) portion of the sample was spiked with internal standards and analysed to confirm the concentration of analytes, prior to spiking, for recoveries and matrix effect analysis.

Quality control

Quality control samples were analysed before and after each batch at three concentrations (10 μg L^{-1}, 100 μg L^{-1}, 500 μg L^{-1}) along with procedural blanks, to ensure the method and instrumental performance and to monitor for contamination of the equipment.

All samples were spiked with the internal standards listed in ESM Table S3, for accurate quantification, and to account for loss from the point of spiking until analysis and to limit matrix effects.

Results and discussion

This paper provides an expanded and broader scope method based on a method published by Petrie et al. [39], enabling the analysis of household and agricultural chemicals whilst utilising one sample preparation protocol and comprehensive UPLC-MS/MS methodology.

UPLC-MS/MS method

All analytes were analysed using MRM and ESI− and ESI+ modes and conditions previously selected by Petrie et al. [39]. Following EU guidelines, two MRM transitions were used for most of the 195 compounds (142 analytes and 53 ISTDs). This is except for cefalexin, ketoprofen, diclofenac, ibuprofen, 1,7-dimethylxanthine and norfluoxetine, which are to be considered as semi-quantitative. For ESI−, the parent ion $[M-H]^-$ was selected, and for ESI+ mode, $[M-H]^+$ was selected. The most abundant daughter ion was used for quantification and the second most for confirmation. ESM Table S2 includes the MRMs for all analytes, the acquisition method was used to analyse them and the assigned internal standard. The MRMs for the internal standards can be found in ESM Table S3.

The reversed-phase BEH C18 column provided good separation and sensitivity for all compounds. These conditions achieved good separation and peak shape for most analytes. Further information can be found in Fig. 1. A more acidic mobile phase was also trialled in the ESI+ method, and although the peak shape and separation for the acidic compounds, particularly the fluoroquinolones, were improved slightly, they only benefitted a small number of compounds and had a detrimental effect against many others. Satisfactory separation of analytes was achieved within a < 40-min retention time.

Quality control criteria as recommended by the EU directive [41] utilised quality control samples, standard tolerances of ion ratio, chromatographic retention time, relative retention time and signal-to-noise ratio.

Instrument performance

The instrument performance was assessed by considering linear response, inter- and intra-day precision and accuracy and, finally, instrumental detection and quantification limits. This data can be found in Table 1.

For the linear response, a linear range covering several orders of magnitude with the r^2 value of ≥ 0.997 was ideal. However, for a few analytes (triclosan, benzoylecgonine and mirtazapine), this could only be achieved through splitting the linear range into two overlapping ranges, each with the r^2 value ≥ 0.997. This allowed adequate quantification across the entire range. Of the 142 compounds, 120 have r^2 values ≥ 0.997. Twenty-one of the remaining compounds have r^2 values ≥ 0.992. Although this is not ideal, it is still adequate for accurate quantification, as the other parameters indicate. Chlorpyrifos has the lowest r^2 value, likely due to the lack of an analogous internal standard; however, it passes further instrumental performance criteria.

The intra-day instrumental performance is high across many compounds. Out of the 142 analytes, the majority of which are very precise, with a deviation of ≤ 5%. The

Table 1 Instrumental performance data for ECs of interest in the mobile phase (ordered by class)

Class of analyte	Analyte	RT	RRT	Linearity		Intra-day instrumental performance		Inter-day instrumental performance		$IDL_{S/N}$ (μg L^{-1})	$IQL_{S/N}$ (μg L^{-1})
				Range (μg L^{-1})	r^2	Precision (deviation) (%)	Accuracy (%)	Precision (%)	Accuracy (%)		
UV filter	Benzophenone-1	9.6	0.9	0.06–684.0	0.996	2.3	106.8	3.3	106.7	0.01	0.06
	Benzophenone-2	7.7	1.0	0.05–583.8	0.997	1.1	99.6	4.2	97.6	0.01	0.05
	Benzophenone-3	21.2	1.2	0.05–404.0	0.995	3.2	84.9	4.5	86.8	0.01	0.05
	Benzophenone-4	6.9	0.9	1.01–502.5	0.997	2.3	103.0	3.8	105.1	0.31	1.01
Parabens	Methylparaben	7.5	1.0	0.06–1122	0.998	1.1	93.3	6.0	97.4	0.01	0.06
	Ethylparaben	8.3	1.0	0.11–663.6	0.997	2.6	112.3	2.1	113.1	0.03	0.11
	Propylparaben	9.2	1.0	0.12–462.0	0.997	5.7	96.4	4.3	98.4	0.04	0.12
	Butylparaben	10.1	1.0	0.06–696.6	0.997	5.0	97.1	3.6	100.3	0.01	0.06
Plasticizer	Bisphenol A	9.0	1.1	0.10–626.4	0.997	2.4	103.6	1.3	104.6	0.03	0.10
Steroid estrogens	E1	9.8	1.0	0.49–989.0	0.998	1.8	96.9	2.1	98.6	0.10	0.49
	E2	9.8	1.0	0.47–949.0	0.997	3.1	96.6	2.6	96.3	0.09	0.47
	EE2	9.7	1.0	0.48–950.0	0.997	2.6	94.6	3.3	93.2	0.10	0.48
Antibiotics and antibacterials	Sulfasalazine	7.1	0.8	0.90–904.0	0.999	3.9	105.2	2.4	104.7	0.27	0.90
	Clarithromycin	18.9	1.1	0.06–561.0	0.999	2.6	99.8	2.4	101.8	0.01	0.06
	Azithromycin	14.0	0.9	0.001–1000	0.998	4.5	108.9	1.5	102.0	0.01	0.05
	Trimethoprim	8.4	1.0	0.10–500.0	0.998	3.0	96.9	2.2	99.5	0.03	0.10
	Sulfamethoxazole	9.6	1.0	0.10–1000	0.999	3.5	95.1	2.4	96.0	0.03	0.10
	Triclosan[a]	12.3	1.2	1.13–225.6112.8–1128	0.997/0.998	9.4	69.1	6.5	71.4	0.34	1.13
	Amoxicillin	3.1	0.2	0.06–439.5	0.995	5.3	105.7	6.7	94.4	0.02	0.06
	Metronidazole	5.3	1.0	1.00–1000	0.999	2.5	105.0	1.2	102.9	0.06	0.21
	Sulfadiazine	4.8	0.9	0.05–795.2	0.999	2.8	105.3	1.5	104.4	0.01	0.03
	Cefalexin[b]	9.2	0.3	15.9–200	0.995	9.5	111.3	12.3	102.9	4.78	15.94
	Ofloxacin	9.6	1.0	0.23–986.0	0.998	4.2	97.4	2.8	95.9	0.07	0.23
	Ciprofloxacin	9.9	1.0	1.18–902	0.999	8.7	89.0	5.5	90.2	0.35	1.18
	Tetracycline	10.0	1.0	0.06–864.0	0.999	6.8	115.1	8.5	113.1	0.02	0.06
	Danofloxacin	10.2	1.0	1.05–1000	0.998	7.3	106.0	6.0	99.2	0.32	1.05
	Oxytetracycline	10.4	1.1	2.36–800.8	0.997	4.6	93.5	3.0	88.9	0.71	2.36
	Chloramphenicol	12.6	0.6	1.74–400	0.999	3.8	103.5	3.0	100.8	0.52	1.74
	Penicillin G	13.1	0.5	4.68–93.6	0.994	10.3	115.5	4.4	111.7	0.02	0.07
	Penicillin V	14.5	0.8	5.00–200	0.993	4.4	88.5	15.0	96.8	0.15	0.49
	Erythromycin	17.2	1.0	204.4–1022	0.999	2.3	94.4	2.9	95.2	0.20	0.65
	Prulifloxacin	18.0	1.9	100–1000	0.997	4.4	98.7	8.9	86.4	2.44	8.13

Table 1 (continued)

Class of analyte	Analyte	RT	RRT	Linearity		Intra-day instrumental performance		Inter-day instrumental performance		$IDL_{S/N}$ (μg L^{-1})	$IQL_{S/N}$ (μg L^{-1})
				Range (μg L^{-1})	r^2	Precision (deviation) (%)	Accuracy (%)	Precision (%)	Accuracy (%)		
	Norfloxacin	9.7	1.0	0.01–1000	0.996	4.1	85.5	4.4	85.1	0.002	0.01
Antifungal	Griseofulvin	17.2	0.9	0.26–205.2	0.999	1.6	89.2	3.0	91.6	0.08	0.26
	Ketoconazole	21.7	1.2	0.02–800.0	0.999	3.8	94.8	2.5	91.7	0.01	0.02
Hypertension	Valsartan	7.6	0.9	1.12–1122	0.998	1.9	115.8	3.5	118.6	0.34	1.12
	Irbesartan	8.6	1.0	0.50–603.6	0.998	2.6	96.9	4.1	98.3	0.10	0.50
	Lisinopril	7.1	0.9	0.93–372.5	0.995	2.2	97.2	7.2	95.2	0.09	0.93
NSAIDs	Ketoprofen[b]	7.9	0.9	0.54–1085	0.998	2.2	99.9	2.6	99.4	0.11	0.54
	Ibuprofen[b]	9.8	1.0	0.05–1071	0.998	2.4	93.7	2.3	94.2	0.01	0.05
	Naproxen	8.1	1.0	0.49–989.0	0.998	1.5	97.7	2.5	98.3	0.10	0.49
	Diclofenac[b]	9.0	1.0	0.10–619.2	0.997	7.9	89.6	4.5	91.8	0.03	0.10
	Acetaminophen	5.1	1.0	0.54–1070	0.998	1.6	97.4	2.6	99.0	0.11	0.54
Lipid regulator	Bezafibrate	7.9	1.0	0.10–976.0	0.998	2.3	97.8	2.8	97.9	0.03	0.10
	Atorvastatin	9.3	1.1	0.05–500.0	0.997	2.6	98.0	3.5	100.9	0.01	0.05
Antihyperlipidaemic	Gemfibrozil	23.3	1.2	1.01–100.5	0.994	7.8	118.5	6.9	121.1	0.11	0.35
Antihyperintensive	Candesartan cilexetil	23.0	0.9	226.8–680.4	0.995	5.2	100.5	0.9	106.9	1.58	5.28
Antihistamine	Fexofenadine	8.4	1.0	0.09–937.5	0.998	2.1	106.3	6.5	104.6	0.03	0.09
	Cetirizine	18.7	1.0	0.08–417.7	0.999	1.3	100.5	1.3	100.8	0.02	0.08
GUD/ED	Sildenafil	18.3	1.0	0.01–1000	1.000	3.5	99.5	3.0	99.1	0.002	0.01
Diabetes	Metformin	2.8	1.0	0.43–862.5	0.998	1.5	96.3	1.3	97.0	0.09	0.43
	Gliclazide	17.8	1.0	0.05–508.0	0.997	2.1	93.2	2.8	95.3	0.01	0.05
	Sitagliptin	11.8	0.7	0.08–646.4	0.998	3.2	111.7	3.0	110.3	0.01	0.02
Cough suppressant	Pholcodine	3.7	0.9	1.14–570.0	0.999	4.7	99.5	3.3	99.2	0.35	1.14
Beta blocker	Atenolol	4.3	1.0	0.10–502.5	0.999	2.1	95.3	2.3	96.8	0.03	0.10
	Metoprolol	11.2	1.0	0.05–507.5	0.999	1.3	96.8	2.0	96.1	0.01	0.05
	Propranolol	15.1	1.0	0.09–434.9	0.999	2.0	105.4	1.0	106.2	0.03	0.09
	Bisoprolol	13.7	0.8	0.10–1004	0.999	4.8	100.4	2.0	96.0	0.0004	0.0012
H_2 receptor agonist	Ranitidine	4.6	1.1	5.17–517.0	0.998	2.5	100.1	9.7	97.4	1.03	5.17
	Cimetidine	5.3	1.0	0.52–1043	0.999	4.2	104.1	9.0	99.3	0.10	0.52
X-ray contrast media	Iopromide	4.9	0.9	5.79–1158	0.997	5.0	101.2	12.0	105.4	1.16	5.79
Various	Buprenorphine	21.8	1.2	0.08–100	0.996	8.9	94.5	11.5	88.2	0.02	0.08
Drug precursor	Ephedrine/pseudoephedrine	7.2	1.0	0.10–500.0	0.997	4.1	94.0	3.4	97.3	0.03	0.10

Table 1 (continued)

Class of analyte	Analyte	RT	RRT	Linearity		Intra-day instrumental performance		Inter-day instrumental performance		$IDL_{S/N}$ (μg L^{-1})	$IQL_{S/N}$ (μg L^{-1})
				Range (μg L^{-1})	r^2	Precision (deviation) (%)	Accuracy (%)	Precision (%)	Accuracy (%)		
	Norephedrine	6.3	0.9	0.50–1000	0.999	4.3	96.3	5.1	95.2	0.01	0.50
Anticancer	Azathioprine	7.8	0.9	0.10–490.0	0.999	7.6	97.5	13.9	97.4	0.03	0.10
	Methotrexate	7.9	1.0	0.92–458.0	0.997	8.7	108.0	4.1	112.2	0.28	0.92
	Ifosfamide	12.7	1.1	0.05–509.0	0.999	2.4	93.6	2.7	95.3	0.01	0.05
	Tamoxifen	22.4	1.0	0.03–668.4	0.998	4.0	96.0	2.4	96.8	0.01	0.03
	Imatinib	15.4	0.8	0.88–88.4	0.994	2.5	103.8	1.5	101.3	0.08	0.28
	Capecitabine	16.1	0.9	0.01–594.6	0.999	2.3	89.2	2.8	89.7	0.001	0.004
	Bicalutamide	18.2	0.9	0.10–784.0	0.995	2.7	90.1	2.9	92.0	0.03	0.10
Anaesthetic and metabolite	Ketamine	10.6	1.0	0.05–500.0	0.998	1.8	92.5	1.3	93.6	0.01	0.05
	Norketamine	11.1	1.0	0.10–500.0	0.999	1.8	94.1	3.2	94.0	0.03	0.10
	Venlafaxine	14.1	1.3	0.04–434.8	0.998	2.5	91.2	1.7	90.5	0.01	0.04
	Desmethylvenlafaxine	10.8	1.0	0.10–500.0	0.998	2.8	101.3	2.1	102.3	0.03	0.10
	Fluoxetine	18.4	1.0	0.05–1000	0.999	1.7	96.8	1.8	98.3	0.01	0.05
	Norfluoxetine[b]	18.4	1.0	0.05 – 500.0	0.998	1.5	102.7	3.1	103.1	0.01	0.05
	Sertraline	19.2	1.0	0.05–500.0	1.000	1.6	95.3	1.7	95.7	0.01	0.05
	Mirtazapine[a]	13.5	1.0	0.05–100.0, 50.0–500.0	0.999/0.997	3.4	94.8	2.7	97.6	0.01	0.05
	Citalopram	15.1	1.0	0.50–1000	0.999	0.7	101.2	2.6	101.8	0.05	0.50
	Desmethylcitalopram	15.2	1.0	0.05–500.0	0.998	1.8	103.0	3.0	103.4	0.01	0.05
	Paroxetine	17.3	0.9	5.00–600	0.998	3.2	103.4	1.3	102.1	0.01	0.03
	Duloxetine	17.8	1.0	1.00–1000	0.997	3.0	91.2	13.6	78.3	0.003	0.01
	Amitriptyline	18.2	1.0	0.11–885.0	1.000	4.5	99.6	2.4	96.8	0.03	0.11
	Nortriptyline	18.4	1.0	0.22–800	0.999	4.0	95.5	3.1	92.9	0.07	0.22
	Norsertraline	19.8	1.0	0.23–100	0.999	8.7	99.0	11.0	91.8	0.07	0.23
Anti-epileptic	Carbamazepine	16.2	1.0	0.05–514.0	1.000	2.0	91.7	1.6	92.7	0.01	0.05
	Carbamazepine-10,11-epoxide	13.5	0.8	0.10–1000	0.997	1.6	88.9	2.1	89.9	0.03	0.10
	10,11-Dihydro-10-hydroxycarbamazepine	13.5	0.8	0.50–100.0	0.997	2.8	92.2	5.6	93.8	0.05	0.50
Calcium channel blocker	Diltiazem	16.7	1.0	0.10–486.2	0.996	2.3	92.7	2.3	93.6	0.01	0.10
	Verapamil	16.2	1.0	0.01–600	0.998	2.9	103.1	2.4	101.9	0.001	0.004
Hypnotic	Temazepam	18.2	1.0	0.05–500.0	0.998	1.0	97.0	1.6	97.9	0.01	0.05
	Oxazepam	17.8	1.0	0.10–800	0.999	3.3	94.8	3.4	94.3	0.02	0.08
	Diazepam	19.5	1.0	0.01–1000	1.000	1.6	100.7	4.5	99.6	0.003	0.01

Table 1 (continued)

Class of analyte	Analyte	RT	RRT	Linearity		Intra-day instrumental performance		Inter-day instrumental performance		$IDL_{S/N}$ (μg L^{-1})	$IQL_{S/N}$ (μg L^{-1})
				Range (μg L^{-1})	r^2	Precision (deviation) (%)	Accuracy (%)	Precision (%)	Accuracy (%)		
Antipsychotic	Quetiapine	17.9	1.0	0.05–1000	0.997	1.4	95.3	1.2	96.4	0.01	0.05
	Risperidone	13.7	0.8	0.01–200	0.997	3.2	101.6	1.2	96.8	0.002	0.01
Dementia	Donepezil	13.9	0.9	0.01–1000	0.998	2.6	110.8	1.3	107.7	0.17	0.58
	Memantine	15.7	1.0	0.05–506.4	0.998	3.5	106.3	0.9	104.3	0.02	0.05
Human indicators	Creatinine	2.7	1.0	1.00–1000	0.999	1.4	100.5	2.8	100.1	0.30	1.00
	Nicotine	3.3	0.8	1.00–500.0	0.998	1.2	98.3	2.4	98.4	0.30	1.00
	Caffeine	8.3	1.2	0.50–500.0	0.999	1.7	99.6	2.8	100.4	0.10	0.50
	Cotinine	7.2	1.0	0.05–1000	0.999	1.5	98.4	1.5	98.8	0.01	0.05
	1,7-Dimethylxanthine[b]	6.8	0.9	1.00–500.0	0.999	6.0	94.3	9.9	94.9	0.30	1.00
Analgesics and metabolites	Morphine	3.5	1.0	1.00–500.0	0.998	2.9	99.1	2.5	97.5	0.30	1.00
	Dihydromorphine	3.3	1.0	0.05–500.0	0.997	4.4	106.0	2.7	108.5	0.01	0.05
	Normorphine	3.4	1.0	1.00–500.0	0.999	1.5	100.9	2.2	99.8	0.30	1.00
	Methadone	17.6	1.0	0.05–400.0	0.998	1.5	98.7	1.4	100.2	0.01	0.05
	EDDP	14.8	1.0	0.05–500.0	0.999	1.2	96.5	1.1	96.4	0.01	0.05
	Codeine	6.1	1.0	0.50–500.0	0.997	2.0	93.5	4.0	95.1	0.10	0.50
	Norcodeine	6.5	1.1	1.00–500.0	0.998	2.8	98.5	4.8	98.6	0.30	1.00
	Dihydrocodeine	5.5	0.9	0.10–500.0	0.999	1.6	94.2	2.1	94.6	0.03	0.10
	Tramadol	11.0	1.0	1.00–500.0	0.999	1.6	100.1	1.9	98.4	0.01	1.00
	N-Desmethyltramadol	11.9	1.1	0.50–500.0	0.998	2.5	92.5	2.2	94.4	0.01	0.50
	O-Desmethyltramadol	8.3	1.2	1.00–400.0	0.997	3.3	95.3	4.9	98.5	0.01	1.00
Stimulants and metabolites	Amphetamine	8.4	1.0	0.10–500.0	0.999	4.4	100.8	1.6	100.7	0.03	0.10
	Methamphetamine	8.5	1.0	0.10–500.0	0.999	2.2	101.0	1.3	101.1	0.03	0.10
	MDMA	8.6	1.0	0.05–1000	0.999	1.3	99.2	1.7	99.8	0.01	0.05
	MDA	8.6	1.0	0.10–1000	0.998	1.1	98.4	0.7	100.0	0.03	0.10
	Cocaine	11.3	1.0	0.05–500.0	0.999	2.2	97.2	1.5	99.0	0.01	0.05
	Benzoylecgonine[a]	9.7	1.0	0.05–100.0, 50.0–500.0	0.998/0.999	2.4	103.4	0.9	103.2	0.01	0.05
	Anhydroecgonine methyl ester	3.5	1.3	0.50–500.0	0.999	2.3	101.1	2.4	98.7	0.10	0.50
	Cocaethylene	12.9	1.0	0.05–500.0	0.999	2.8	95.1	1.7	94.7	0.01	0.05
	Mephedrone	9.8	1.0	0.05–500.0	0.998	1.8	87.1	2.9	85.7	0.01	0.05
	MDPV	12.1	0.9	0.05–500.0	0.999	2.2	99.6	0.7	101.4	0.01	0.05
	Heroin	10.9	1.0	0.50–500.0	0.999	1.9	98.2	1.8	99.3	0.10	0.50

Table 1 (continued)

Class of analyte	Analyte	RT	RRT	Linearity		Intra-day instrumental performance		Inter-day instrumental performance		$IDL_{S/N}$ (μg L^{-1})	$IQL_{S/N}$ (μg L^{-1})
				Range (μg L^{-1})	r^2	Precision (deviation) (%)	Accuracy (%)	Precision (%)	Accuracy (%)		
Opioid and metabolite	6-Acetylmorphine	7.7	1.1	0.10–500.0	0.997	6.1	95.3	5.1	100.1	0.03	0.10
Pesticides, fungicides and herbicides	Thiamethoxam	8.3	0.4	1.00–100	0.994	4.7	93.8	5.4	96.9	0.02	0.06
	Imidacloprid	10.1	0.6	0.10–595.2	0.996	2.8	100.5	5.5	103.5	0.01	0.04
	Clothianidin	10.4	0.5	1.00–800	0.999	3.2	97.9	3.3	98.6	0.01	0.04
	Metazachlor	17.1	1.0	0.05–1011	0.999	2.5	106.0	2.6	104.7	0.004	0.01
	Terbuthylazine	19.3	1.0	0.05–519	1.000	2.4	99.8	3.3	97.5	0.01	0.02
	Methiocarb	19.4	1.0	0.08–1007	0.999	1.9	101.8	1.8	100.6	0.02	0.08
	Dichlofluanid	20.4	1.1	6.83–1092	0.994	3.8	94.9	4.4	90.9	1.29	4.30
	Flufenacet	20.5	1.2	0.01–986.0	0.997	2.0	104.2	2.9	106.2	0.002	0.01
	Oxadiazon	24.2	1.2	1.00–99.6	0.996	4.0	95.5	2.8	97.1	0.02	0.08
	Chlorpyrifos[c]	24.8	1.5	1.87–98.5	0.985	11.8	80.7	7.8	83.3	0.56	1.87
	Triallate	24.9	1.3	0.03–79.0	0.992	7.6	81.3	13.2	70.6	0.01	0.03
Veterinary pharmaceuticals	Tylosin	17.3	1.0	0.56–560.0	0.999	2.2	99.5	4.0	100.2	0.11	0.56
	Sulfapyridine	6.4	1.2	0.05–800	0.999	2.6	110.7	1.1	109.5	0.01	0.03
	Sarafloxacin	10.9	0.7	0.88–442	0.995	5.2	112.1	2.3	107.1	0.22	0.75
	Ceftiofur	12.1	1.3	0.28–800.0	0.993	3.6	89.5	2.0	86.4	0.08	0.28
	Diazinon	21.9	1.2	0.11–2100	0.998	2.7	98.9	4.1	96.0	0.01	0.02

Where possible, instrumental performance was determined at concentrations of 10 μg L^{-1}, 100 μg L^{-1} and 500 μg L^{-1}; i.e. those analytes where these concentrations were outside the range of linearity or results were <LOQ were not included

IDL instrumental detection limit, *IQL* instrumental quantification limit

[a] Linear range was split into two overlapping ranges to ensure the r^2 value ≥ 0.997

[b] Semi-quantitative, due to only one MRM transition

[c] Semi-quantitative, due to poor r^2 value

precision of 20 compounds was between 5 and 10%, and only one compound was > 10% (chlorpyrifos (11.8%), likely due to poor r^2). However, all deviations are inside the recommended maximum of 20%. For 119/142 compounds, the accuracy lay within the ideal range of 90–110%. Of the remaining compounds, only triclosan has an inaccuracy of > 20%.

Regarding the inter-day instrumental performance, the precision is high across many analytes with 115 analytes with a deviation of ≤ 5%. Of the 27 analytes with > 5% deviation, only 8 were > 10%. The inter-day accuracy of these compounds was also high, with only 20 compounds that deviated from the QC by > 10%, 4 of which were only slightly greater than 20%. Data for both intra- and inter-day precision and accuracy can be found ESM Table S7.

IDLs ranged from 0.4 ng L^{-1} (bisoprolol) to 4783 ng L^{-1} (cefalexin), and IQLs ranged from 1.2 ng L^{-1} (bisoprolol) to 15,940 ng L^{-1} (cefalexin). Whilst many of these IQL concentrations are very low, the samples may still need to be concentrated (utilising SPE), as the concentrations of most compounds in environmental matrices are likely to be even lower.

Method performance

Sample extraction was carried out using SPE following the method shown in Fig. 1 (sections "Extraction for liquid matrices" and "Extraction for solid matrices") and has shown good extraction performance for many CECs. The Oasis HLB sorbent is essential to multi-residue methods, as it has the ability to retain a large range of polar analytes at neutral pH and is therefore widely used in analysis of environmental matrices. However, two drawbacks have been found in the use of this sorbent. Firstly, HLB might result in low recovery of very polar compounds such as metformin and creatinine. This is easily remedied, as metformin and creatinine along with acetaminophen, nicotine, caffeine and 1,7-dimethylxanthine are present in the environment as such high levels that direct injection are utilised instead. The second drawback of HLB is its lack of selectivity. Whilst enabling the extraction of a large variety of polar analytes, in complex matrices, much of the matrix can be extracted along with the chosen analytes, causing significant signal interference. It is therefore important to assess matrix suppression. Previously, it was found that the use of HLB with digested solids provided poor results. This not only necessitated the use of an alternative sorbent, MCX, but also splitting the eluents into acidic and basic fractions (see Fig. 1) [39].

Figure 2 shows relative recoveries for all matrices: surface water, effluent, influent, SPM and digested solids. These very different matrices exhibit similar ranges of relative recovery. Most of the compounds had recoveries between 80 and 120%. Due to the complexity of environmental matrices, not all compounds were recovered adequately across all matrices; therefore, the number of compounds accurately quantifiable in each matrix varies from 138 analytes in surface water to 96 analytes in digested solids. The data for relative and absolute recoveries for all matrices can be found in ESM Tables S8 and S9 and Fig. S2.

Matrix suppression was analysed for liquid matrices (see Fig. 3 and ESM Table S10). Proximity to zero shows limited matrix effects. Most analytes experienced matrix suppression, shown in Fig. 3 as a positive percentage. For all matrices, at least 108 analytes were below 70% suppression. However, a few compounds, primarily from ESI−, experienced significant (> 20%) signal enhancement. Some compounds experienced signal enhancement in some matrices and suppression in others (naproxen, erythromycin, EE2, bicalutamide, candesartan cilexetil, gemfibrozil, chlorpyrifos). This considerable variation is to be expected for a multi-residue method with this variety of analytes. It especially highlights the importance of using relevant analogous internal standards, as well as thoroughly exploring the different matrices relevant to this work.

The method accuracy of the method was 107.0%, and the precision was 13.4%. MQLs for liquid matrices range from 0.004 ng L^{-1} for bisoprolol, in surface water, to 3118 ng L^{-1} for creatinine, in influent (Table 2). For the remaining analytes, there is not enough environmental data on their presence in the UK to comment on whether the MDLs and MQLs are low enough to measure these levels in the catchment of interest. Therefore, this method was applied to five different matrices at a WwTW in the South West UK, which were influent, effluent, surface water, SPM and digested solids, to underline the utilisation of this method to relevant environmental matrices.

Overall, this method provides a clear benefit when used in a catchment-based study, compared to more complex approaches with multiple sample preparation and analytical methods for different classes of CECs from the same matrices. The main advantage of the multi-residue method presented in this paper is the applicability of this method to liquid and solid matrices. In particular, SPM, which is an often forgotten but critical aspect of wastewater, as it contains many more hydrophobic compounds at significant concentrations within this matrix [28, 42, 43].

Application to environmental matrices

The data is presented as average concentrations with variation across a week (Table 3 and ESM Tables S11–S15). As expected, the liquid influent fraction shows the highest variation in concentrations both across the week and between different CECs. Of the 138 analytes of this method, 70% were quantified in influent at this site. Creatinine had the highest average concentration of any analyte in this study (1.3 ± 0.3 mg L^{-1}) and is often used a human indicator due to its correlation to population [44, 45]. Other human indicators were present at high levels throughout the week. The other CECs were present at a range of concentrations. Methiocarb, for example, is present intermittently across the week with an average concentration of 3.7 ± 0.6 ng L^{-1}, which is very close to its MQL of 0.86 ng L^{-1}. However, acetaminophen reaches concentrations several magnitudes higher

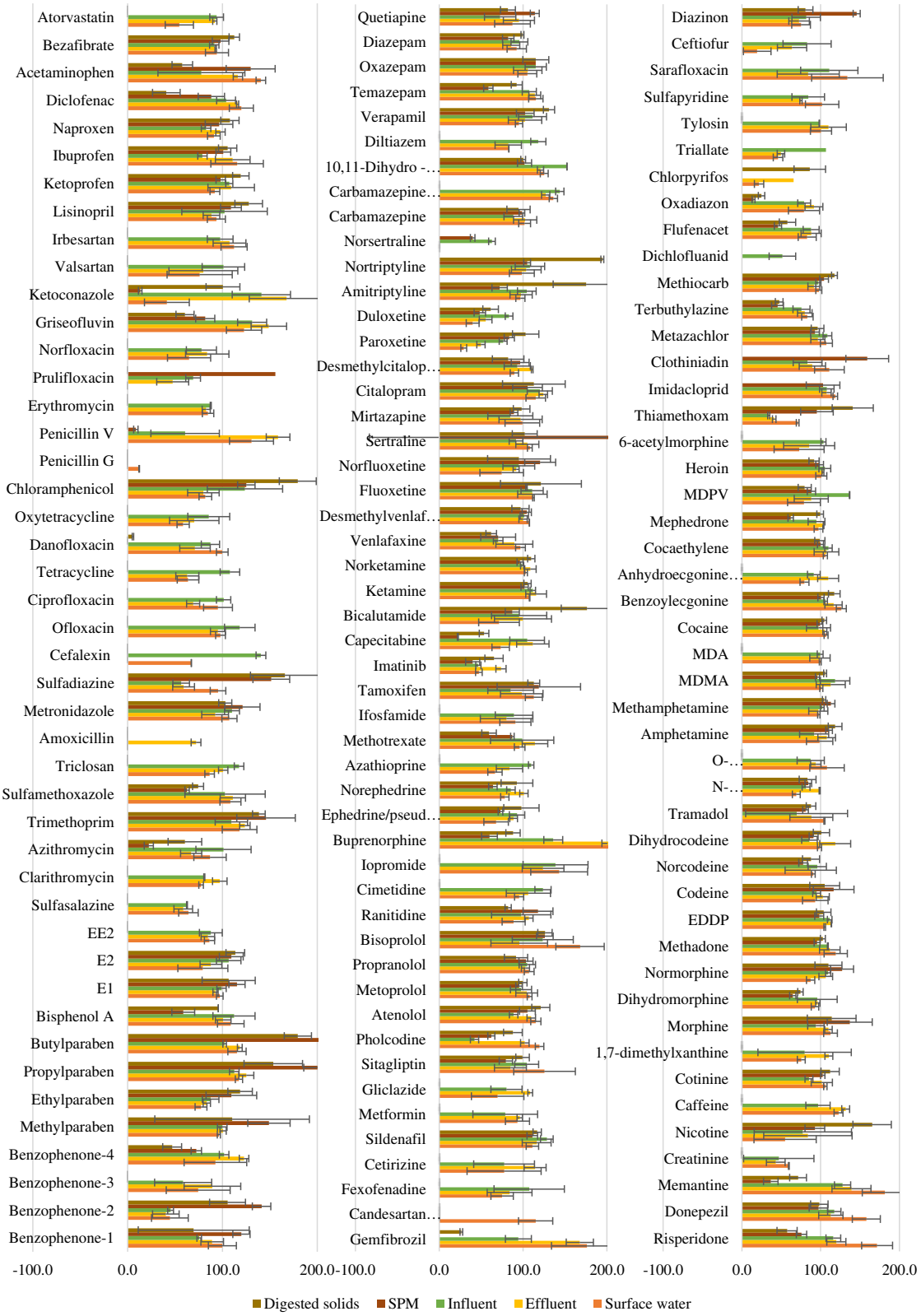

Fig. 2 Relative recoveries for all matrices. Error bars show the range of standard deviation

than methiocarb, at 393.6 ± 100.0 μg L^{-1}. This is slightly higher than those published in a review by Verlicchi et al. [11] which had an absolute highest concentration of 246 μg L^{-1}. It is interesting to see the presence of the (pesticide) methiocarb in

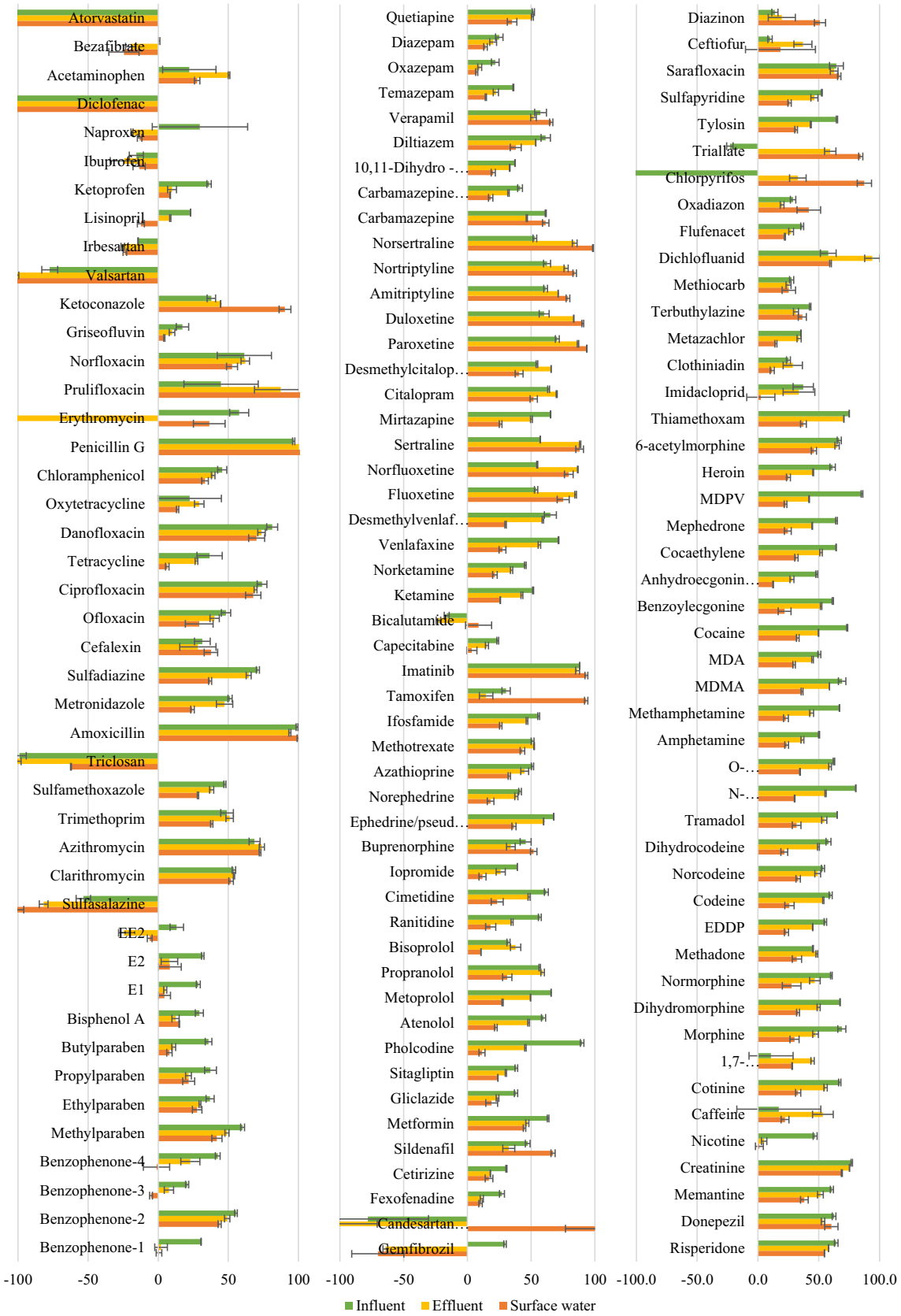

Fig. 3 Matrix suppression for all liquid matrices. Error bars show the range of standard deviation

Table 2 Method performance data for ECs of interest in the mobile phase (ordered by class)

Class of analyte	Analyte	Surface water (ng L^{-1})		Effluent (ng L^{-1})		Influent (ng L^{-1})		Solid particulate matter (ng g^{-1})		Digested solids (ng g^{-1})	
		MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL
UV filter	Benzophenone-1	0.07	0.35	0.14	0.71	0.23	1.15	0.004	0.02	0.14	0.70
	Benzophenone-2	0.16	0.79	0.34	1.68	0.36	1.82	0.004	0.02	0.09	0.44
	Benzophenone-3	0.15	0.77	0.19	0.97	0.37	1.87	–	–	–	–
	Benzophenone-4	2.09	6.90	5.78	19.1	7.83	25.8	0.21	0.70	4.01	13.2
Parabens	Methylparaben	0.08	0.40	0.19	0.94	0.28	1.41	0.003	0.02	0.06	0.31
	Ethylparaben	0.24	0.79	0.46	1.52	0.49	1.61	0.01	0.05	0.17	0.57
	Propylparaben	0.25	0.83	0.47	1.54	0.63	2.08	0.01	0.03	0.22	0.72
	Butylparaben	0.08	0.38	0.14	0.71	0.24	1.21	0.002	0.01	0.10	0.52
Plasticizer	Bisphenol A	0.26	0.86	0.56	1.84	0.85	2.79	0.03	0.09	0.27	0.88
Steroid estrogens	E1	0.78	3.92	0.15	7.69	1.96	9.78	0.04	0.21	1.68	8.38
	E2	0.90	4.48	1.41	7.03	1.84	9.22	0.04	0.21	1.48	7.41
	EE2	0.98	4.91	1.46	7.32	1.83	9.15	–	–	–	–
Antibiotics and antibacterials	Sulfasalazine	4.31	14.2	9.66	31.9	12.6	41.4	–	–	–	–
	Clarithromycin	0.18	0.90	0.28	1.40	0.34	1.69	–	–	–	–
	Azithromycin	0.08	0.26	0.21	0.68	0.14	0.45	0.03	0.10	0.01	0.04
	Trimethoprim	0.26	0.85	0.51	1.67	0.73	2.41	0.01	0.03	0.07	0.22
	Sulfamethoxazole	0.19	0.63	0.47	1.56	0.72	2.38	0.02	0.08	0.12	0.41
	Triclosan	2.93	9.68	4.55	15.0	4.93	16.3	–	–	–	–
	Amoxicillin	–	–	0.26	0.86	–	–	–	–		
	Metronidazole	0.29	0.98	0.68	2.27	0.57	1.90	0.03	0.09	0.03	0.10
	Sulfadiazine	0.05	0.18	0.18	0.59	0.18	0.62	0.003	0.01	0.003	0.01
	Cefalexin	35.6	118.7	10.2	33.9	18.9	63.1	–	–	–	–
	Ofloxacin	0.35	1.17	0.72	2.40	0.58	1.93	–	–	–	–
	Ciprofloxacin	1.85	6.17	5.10	17.0	3.48	11.6	–	–	–	–
	Tetracycline	0.15	0.50	0.30	1.01	0.18	0.59	–	–	–	–
	Danofloxacin	1.58	5.28	4.45	14.85	3.62	12.08	–	–	2.84	9.45
	Oxytetracycline	6.04	20.1	10.1	33.6	8.26	27.5	–	–	–	–
	Chloramphenicol	3.18	10.6	6.52	21.7	4.21	14.0	0.21	0.69	0.15	0.48
	Penicillin G	0.89	2.98	–	–	–	–	–	–	–	–
	Penicillin V	0.56	1.86	0.92	3.06	2.40	8.00	0.84	2.80	–	–
	Erythromycin	1.15	3.83	2.35	7.85	2.22	7.41	–	–	–	–
	Prulifloxacin	–	–	51.3	171.0	35.3	117.6	–	–	–	–
	Norfloxacin	0.01	0.04	0.02	0.06	0.02	0.07	–	–	–	–
Antifungal	Griseofulvin	0.32	1.06	0.52	1.74	0.59	1.98	0.05	0.16	0.06	0.21
	Ketoconazole	0.06	0.21	0.03	0.10	0.04	0.12	0.02	0.07	0.00	0.01
Hypertension	Valsartan	2.81	9.26	6.40	21.1	7.24	23.9	–	–	–	–
	Irbesartan	0.89	4.47	1.88	9.38	2.50	12.5	–	–	–	–
	Lisinopril	2.17	21.7	4.25	42.5	3.25	32.5	0.04	0.43	0.25	2.47
NSAIDs	Ketoprofen	0.74	3.72	1.60	8.00	2.38	11.9	0.06	0.28	0.47	2.35
	Ibuprofen	0.06	0.31	0.08	0.42	0.19	0.93	0.005	0.02	0.07	0.36
	Naproxen	0.61	3.07	1.17	5.85	6.29	31.5	0.05	0.25	0.60	3.02
	Diclofenac	0.22	0.73	0.44	1.44	0.67	2.22	0.02	0.06	0.75	2.46
	Acetaminophen	1.20	6.02	2.39	12.0	138.0*	1017*	0.04	0.21	2.74	13.7
Lipid regulator	Bezafibrate	0.22	0.66	0.38	1.25	0.64	2.11	0.02	0.05	0.18	0.60
	Atorvastatin	0.14	0.70	0.17	0.84	0.17	0.85	–	–	–	–
Antihyperlipidemic	Gemfibrozil	0.30	1.00	0.63	2.11	1.12	3.75	–	–	0.20	0.67

Table 2 (continued)

Class of analyte	Analyte	Surface water (ng L^{-1})		Effluent (ng L^{-1})		Influent (ng L^{-1})		Solid particulate matter (ng g^{-1})		Digested solids (ng g^{-1})	
		MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL
Antihyperintensive	Candesartan Cilexetil	6.89	23.0	–	–	–	–	–	–	–	–
Antihistamine	Fexofenadine	0.21	0.69	0.40	1.32	0.56	1.85	–	–	–	–
	Cetirizine	0.26	0.87	0.32	1.06	0.52	1.72	–	–	–	–
GUD/ED	Sildenafil	0.01	0.03	0.02	0.05	0.01	0.05	0.001	0.003	0.001	0.003
Diabetes	Metformin	156.0*	515.0*	163.0*	460.0*	457.0*	1509*	–	–	–	–
	Gliclazide	0.15	0.77	0.16	0.82	0.22	1.09	–	–	–	–
	Sitagliptin	0.03	0.09	0.08	0.27	0.06	0.22	0.004	0.01	0.003	0.01
Cough suppressant	Pholcodine	2.25	7.42	8.02	26.5	25.3	83.3	0.28	0.92	1.52	5.00
Beta blocker	Atenolol	0.20	0.66	0.56	1.84	0.71	2.35	0.01	0.05	0.10	0.33
	Metoprolol	0.07	0.35	0.19	0.96	0.28	1.40	0.01	0.03	0.03	0.14
	Propranolol	0.29	0.96	0.73	2.41	0.68	2.25	0.01	0.04	0.13	0.42
	Bisoprolol	0.001	0.004	0.004	0.01	0.003	0.01	0.0001	0.0005	0.0001	0.0005
H_2 receptor agonist	Ranitidine	7.96	39.8	22.3	111.4	14.8	73.8	0.44	2.19	4.81	24.1
	Cimetidine	1.60	7.98	3.12	15.6	5.06	25.3	–	–	–	–
X-ray contrast media	Iopromide	5.97	29.9	14.1	70.6	24.5	123.0	–	–	–	–
Various	Buprenorphine	0.06	0.20	0.11	0.36	0.18	0.61	0.02	0.07	0.01	0.05
Drug precursor	Ephedrine/pseudoephedrine	0.60	1.97	1.62	5.36	1.32	4.36	0.02	0.07	0.11	0.35
	Norephedrine	0.18	8.82	0.35	17.3	0.37	18.6	0.01	0.39	0.04	1.85
Anticancer	Azathioprine	0.17	0.55	0.36	1.20	0.41	1.36	–	–	–	–
	Methotrexate	6.13	20.2	9.04	29.8	7.11	23.5	0.16	0.53	1.64	5.42
	Ifosfamide	0.08	0.40	0.24	1.22	0.31	1.53	–	–	–	–
	Tamoxifen	14.5	72.6	0.76	3.82	0.70	3.50	0.004	0.01	2.23	11.14
	Imatinib	0.88	2.93	1.13	3.76	1.78	5.95	0.10	0.35	0.06	0.21
	Capecitabine	0.01	0.02	0.01	0.03	0.01	0.03	0.002	0.01	0.001	0.003
	Bicalutamide	0.22	0.72	0.31	1.02	0.32	1.07	0.02	0.06	0.01	0.03
Anaesthetic and metabolite	Ketamine	0.07	0.37	0.19	0.93	0.24	1.20	0.005	0.02	0.03	0.17
	Norketamine	0.23	0.76	0.56	1.86	0.72	2.37	0.02	0.05	0.10	0.33
	Venlafaxine	0.07	0.37	0.24	1.20	0.37	1.83	0.01	0.03	0.08	0.38
	Desmethylvenlafaxine	0.24	0.80	0.66	2.18	0.85	2.79	0.01	0.05	0.09	0.29
	Fluoxetine	1.14	5.71	1.42	7.08	0.50	2.52	0.005	0.02	0.11	0.53
	Norfluoxetine	1.64	8.21	1.27	6.35	0.42	2.12	0.004	0.02	0.14	0.68
	Sertraline	1.61	8.07	1.21	6.05	0.74	3.72	0.002	0.01	0.17	0.86
	Mirtazapine	0.09	0.44	0.25	1.25	0.39	1.94	0.01	0.03	0.05	0.27
	Citalopram	0.61	6.08	1.41	14.1	1.24	12.4	0.02	0.24	0.16	1.64
	Desmethylcitalopram	0.14	0.69	0.36	1.82	0.31	1.54	0.01	0.03	0.05	0.24
	Paroxetine	0.18	0.59	0.21	0.69	0.13	0.45	0.01	0.02	0.005	0.02
	Duloxetine	0.04	0.13	0.05	0.18	0.04	0.12	0.003	0.01	0.002	0.01
	Amitriptyline	0.16	0.55	0.33	1.09	0.30	1.02	0.02	0.07	0.01	0.03
	Nortriptyline	0.33	1.11	0.63	2.11	0.61	2.03	0.03	0.10	0.02	0.06
	Norsertraline	–	–	–	–	1.07	3.58	0.09	0.28	–	–
Anti-epileptic	Carbamazepine	0.08	0.38	0.19	0.93	0.27	1.37	0.01	0.03	0.10	0.48
	Carbamazepine-10,11-epoxide	0.16	0.53	0.55	1.82	0.53	1.76	–	–	–	–
	10,11-Dihydro-10-hydroxycarbamazepine	0.34	3.37	0.84	8.41	0.99	9.94	0.02	0.25	0.43	4.35
Calcium channel blocker	Diltiazem	0.11	1.11	0.32	3.23	0.27	2.68	–	–	–	–
	Verapamil	0.01	0.02	0.01	0.04	0.01	0.03	0.001	0.002	0.0004	0.001

Table 2 (continued)

Class of analyte	Analyte	Surface water (ng L^{-1})		Effluent (ng L^{-1})		Influent (ng L^{-1})		Solid particulate matter (ng g^{-1})		Digested solids (ng g^{-1})	
		MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL
Hypnotic	Temazepam	0.08	0.38	0.14	0.69	0.18	0.92	0.01	0.04	0.16	0.82
	Oxazepam	0.11	0.36	0.22	0.72	0.20	0.66	–	–	0.01	0.03
	Diazepam	0.02	0.06	0.04	0.13	0.04	0.12	0.002	0.01	0.002	0.01
Antipsychotic	Quetiapine	0.10	0.48	0.21	1.07	0.26	1.32	0.004	0.02	0.05	0.26
	Risperidone	0.01	0.02	0.02	0.06	0.02	0.06	0.001	0.004	0.002	0.01
Dementia	Donepezil	0.55	1.83	1.54	5.12	1.48	4.93	0.09	0.30	0.09	0.29
	Memantine	0.04	0.14	0.11	0.36	0.12	0.39	0.02	0.07	0.01	0.04
Human indicators	Creatinine	511*	1686*	771*	2544*	945*	3118*	–	–	–	–
	Nicotine	3.34	11.0	5.44	18.0	508*	2296*	0.16	–	0.66	2.19
	Caffeine	0.37	1.83	1.11	5.57	121*	581*	–	–	–	–
	Cotinine	0.07	0.35	0.21	1.06	0.27	1.34	0.005	0.02	0.24	1.22
	1,7-Dimethylxanthine	3.19	10.5	11.4	37.6	560*	2165*	–	–	–	–
Analgesics and metabolites	Morphine	2.65	8.75	6.34	20.9	8.85	29.2	0.11	0.37	1.92	6.33
	Dihydromorphine	0.11	0.55	0.32	1.59	0.05	2.51	0.01	0.04	0.09	0.45
	Normorphine	3.54	11.7	7.84	25.9	9.99	33.0	0.12	0.39	1.74	5.75
	Methadone	0.11	0.54	0.21	1.04	0.20	1.01	0.01	0.03	0.03	0.17
	EDDP	0.21	1.05	0.29	1.47	0.23	1.13	0.01	0.03	0.04	0.20
	Codeine	0.74	3.71	1.46	7.31	2.56	12.8	0.04	0.21	0.33	1.66
	Norcodeine	2.88	9.52	8.32	27.4	8.53	28.2	0.19	0.64	1.26	4.17
	Dihydrocodeine	0.23	0.75	0.55	1.83	0.88	2.89	0.02	0.05	0.11	0.36
	Tramadol	0.08	8.20	0.21	21.3	0.30	30.0	0.01	0.62	0.03	3.26
	N-Desmethyltramadol	0.12	5.92	0.30	15.0	0.56	27.9	0.01	0.30	0.04	2.02
	O-Desmethyltramadol	0.09	8.53	0.28	27.8	0.31	31.4	–	–	–	–
Stimulants and metabolites	Amphetamine	0.68	2.23	1.11	3.65	1.23	4.07	0.01	0.05	0.09	0.29
	Methamphetamine	0.32	1.05	0.71	2.35	0.95	3.13	0.01	0.04	0.09	0.30
	MDMA	0.10	0.50	0.27	1.35	0.34	1.70	0.01	0.03	0.04	0.18
	MDA	0.53	1.74	1.00	3.30	0.99	3.26	–	–	–	–
	Cocaine	0.07	0.35	0.22	1.11	0.46	2.31	0.01	0.03	0.03	0.15
	Benzoylecgonine	0.07	0.34	0.18	0.91	0.21	1.07	0.005	0.02	0.03	0.14
	Anhydroecgonine methyl ester	0.93	4.67	1.99	9.96	2.95	14.8	–	–	–	–
	Cocaethylene	0.07	0.35	0.21	1.04	1.31	6.54	0.01	0.03	0.03	0.17
	Mephedrone	0.22	1.09	0.44	2.19	0.55	2.75	0.01	0.04	0.06	0.31
	MDPV	0.04	0.22	0.12	0.59	0.48	2.41	0.01	0.03	0.04	0.20
Opioid and metabolite	Heroin	0.92	4.62	3.44	17.2	4.18	20.9	0.05	0.25	0.56	2.79
	6-Acetylmorphine	0.28	0.94	0.76	2.50	0.89	2.95	–	–	–	–
Pesticides, fungicides and herbicides	Thiamethoxam	0.13	0.42	0.44	1.46	0.53	1.76	0.01	0.03	0.01	0.02
	Imidacloprid	0.04	0.15	0.10	0.33	0.10	0.33	0.01	0.02	–	–
	Clothianidin	0.06	0.19	0.14	0.47	0.15	0.50	0.004	0.01	–	–
	Metazachlor	0.02	0.06	0.04	0.14	0.04	0.13	0.002	0.01	0.002	0.01
	Terbuthylazine	0.03	0.11	0.07	0.22	0.07	0.23	0.01	0.02	0.01	0.02
	Methiocarb	0.13	0.43	0.27	0.91	0.26	0.86	0.01	0.04	0.01	0.04
	Dichlofluanid	–	–	–	–	25.2	83.8	–	–	–	–
	Flufenacet	0.01	0.04	0.02	0.07	0.02	0.07	0.002	0.01	0.002	0.01
	Oxadiazon	0.15	0.49	0.26	0.85	0.30	0.98	0.08	0.26	0.05	0.16
	Chlorpyrifos	12.9	42.9	8.54	28.5	–	–	–	–	0.33	1.09
	Triallate	0.11	0.37	0.20	0.68	0.09	0.31	–	–	–	–

Table 2 (continued)

Class of analyte	Analyte	Surface water (ng L^{-1})		Effluent (ng L^{-1})		Influent (ng L^{-1})		Solid particulate matter (ng g^{-1})		Digested solids (ng g^{-1})	
		MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL	MDL	MQL
Veterinary pharmaceuticals	Tylosin	1.28	6.39	2.23	11.1	3.27	16.3	–	–	–	–
	Sulfapyridine	0.04	0.14	0.11	0.37	0.10	0.33	–	–	–	–
	Sarafloxacin	0.83	2.78	2.66	8.86	2.01	6.72	–	–	–	–
	Ceftiofur	2.17	7.23	1.32	4.41	1.02	3.39	–	–	–	–
	Diazinon	0.03	0.11	0.07	0.23	0.06	0.21	0.00	0.01	0.003	0.01

*Calculated for direct injection

influent, as this is generally used for plant protection, particularly against slugs. It is thought to be an unlikely compound to make its way into influent and thought to enter surface waters via direct application to the environment. Its presence in the sewage treatment works, although in a low concentration, is notable as it may indicate incorrect disposal, although a higher concentration would be expected from this. It was recently banned for use as a molluscicide in 2014, the grace period of which ended the month before these samples were collected; however, it could still be used as an insect repellent and seed treatment [46]. Other pesticides found in influent at this site include imidacloprid and flufenacet, which are more widely used for vegetable and fruit crops and may be due to rinsing of food prior to consumption. However, this needs further detailed investigation.

Of further interest is the presence of veterinary pharmaceuticals in wastewater, and the presence of these can be justified by considering the number of household pets and the possible disposal route of pet waste down the lavatory. However, it is interesting to note that sulfapyridine (1339.5 ± 330.3 ng L^{-1}) and ceftiofur (451.8 ± 129.2 ng L^{-1}) are usually reserved for the use with individual pigs and cows. These levels suggest potential herd applications, incorrect disposal or unknown contribution of livestock wastewater to this WwTW. Further investigation is needed to determine the source and persistence of these levels.

For the solid portion of influent (SPM), 64% of the 98 analytes quantifiable with this method were found. Only a small fraction of the total concentration can be found in the SPM, as most CECs, particularly pharmaceuticals, prefer to partition to the aqueous phase. For example, only a fraction of the total concentration (6.4%) of bisphenol A (3.64 log K_{ow}) appears in SPM. However, in this case, the concentrations are so high in influent that this results in bisphenol A contributing a large portion of the total measurable concentration of CECs. Concentrations range from an average concentration of 0.1 ng L^{-1} to 1383 ng L^{-1} in SPM (converted to ng L^{-1} for simple comparison to influent concentrations). Ketoconazole prefers to partition to the solid phase with 31% higher average concentration present in SPM. This is not surprising when considering its log K_{ow} of 4.45. The antidepressants fluoxetine (4.65 log K_{ow}) and amitriptyline (4.95 log K_{ow}) partition partially with SPM concentrations at 82% and 73% to that in influent. The results for fluoxetine are in line with the results published by Baker and Kasprzyk-Hordern [47]. However, sorption of amitriptyline is far higher in this study. This to be expected, as influent is highly variable, and many factors can affect sorption to solids. Therefore, it is important to analyse both the liquid and solid compartments of this matrix.

There are fewer CECs in effluent than in influent. Of the 137 analytes quantifiable in effluent, 62% were found at the WwTW. Generally, these results show lower concentrations after treatment. However, imatinib, *O*-desmethyltramadol, carbamazepine and its epoxide metabolite and venlafaxine and its metabolite increase in effluent. For metabolites, this may be due to degradation/metabolism of the parent compound during treatment. For the parent compounds, this may be due to the undetected presence of conjugated metabolites in influent transforming back to the parent compound. This phenomenon requires more detailed investigation. Comparison of the influent and effluent concentrations can provide data on treatment efficiencies. Trickling filters are employed at the WwTW for the treatment of influent. The results show poor removal of imidacloprid, tramadol, *N*-desmethyltramadol, bicalutamide, ranitidine, cetirizine and fexofenadine. Acetaminophen, on the other hand, shows high removal of 99%, similar to what is often seen in the literature for conventional activated sludge treatment [11, 48]. Caffeine, its metabolite 1,7-dimethylxanthine and metformin are highly removed but still at high concentrations of 2.9 ± 0.4 μg L^{-1}, 8.4 ± 1.1 μg L^{-1} and 14.1 ± 0.9 μg L^{-1}, respectively.

In the surface waters downstream of the WwTW, 51% of the 138 analytes that can be quantified are in this matrix. Of particular noteworthiness is the antidiabetic metformin, which is found at levels > 3000 ng L^{-1}. Metformin has very high usage, and after administration, 100% of the dose is excreted unchanged [49]. Caldwell et al. [50] calculated the PNEC of metformin to be 1 mg L^{-1}, based on critical evaluation of previously published work. This suggests that despite the high environmental levels, metformin is currently of low risk to this catchment. Within the literature, many compounds can be found to have different PNECs due to a lack of consistency in (a) assessment factors

Table 3 Weekly average concentrations of ECs found in several matrices at site A (mean concentration ± variation from mean across the week)

Class of analyte	Compound	Surface water (ng L^{-1})	Effluent (ng L^{-1})	Influent (ng L^{-1})	SPM (ng L^{-1})	Digested solids (ng g^{-1})
UV filter	Benzophenone-1	<MQL	<MQL	949.2 ± 1134.5	13.8 ± 12.0	N/A
	Benzophenone-2	<MQL	62.0 ± 36.0	1701.0 ± 2176.6	22.0 ± 25.9	10.2 ± 5.2
	Benzophenone-3	19.0 ± 1.9	96.7 ± 36.7	2051.5 ± 897.3	N/A	N/A
	Benzophenone-4	607.9 ± 344.8	4224.7 ± 3455.7	15,817.4 ± 13,404.6	9.9 ± 4.0	<MQL
Parabens	Methylparaben	7.0 ± 2.5	42.3 ± 49.4	23,401.4 ± 36,282.5	122.1 ± 99.8	360.2 ± 132.5
	Ethylparaben	<MQL	<MQL	2166.0 ± 2427.8	21.0 ± 17.8	<MQL
	Propylparaben	3.7 ± 0.7	32.1 ± 11.4	5456.2 ± 3190.2	33.2 ± 32.6	<MQL
	Butylparaben	<MQL	<MQL	337.4 ± 434.5	40.3 ± 66.7	<MQL
Plasticizer	Bisphenol-A	38.1 ± 15.1	473.6 ± 256.2	20,395.5 ± 15,740.7	1383.9 ± 1204.0	4365.6 ± 309.6
Steroid estrogens	E1	<MQL	6.3 ± 1.6	47.0 ± 10.7	<MQL	41.5 ± 2.9
	E2	<MQL	<MQL	<MQL	<MQL	<MQL
	EE2	<MQL	<MQL	<MQL	N/A	N/A
Antibiotics and antibacterials	Sulfasalazine	53.6 ± 6.8	241.4 ± 27.5	612.9 ± 248.7	N/A	N/A
	Clarithromycin	102.0 ± 26.8	776.2 ± 121.4	1321.1 ± 293.4	N/A	N/A
	Azithromycin	3.9 ± 3.2	108.7 ± 48.4	793.1 ± 256.1	<MQL	5.7 ± 5.5
	Trimethoprim	25.9 ± 5.7	175.6 ± 30.2	626.2 ± 151.9	16.7 ± 6.3	11.1 ± 2.8
	Sulfamethoxazole	33.2 ± 3.1	54.3 ± 6.2	126.7 ± 58.6	<MQL	<MQL
	Triclosan	<MQL	220.9 ± 52.2	2480.5 ± 801.6	N/A	N/A
	Amoxicillin	N/A	<MQL	N/A	N/A	N/A
	Metronidazole	10.1 ± N/A	37.0 ± 6.5	81.6 ± 23.6	<MQL	<MQL
	Sulfadiazine	<MQL	<MQL	<MQL	8.5 ± 3.3	<MQL
	Cefalexin	N/A	N/A	N/A	N/A	N/A
	Ofloxacin	71.5 ± 29.5	108.7 ± 52.7	<MQL	N/A	N/A
	Ciprofloxacin	N/A	<MQL	N/A	N/A	N/A
	Tetracycline	<MQL	<MQL	251.5 ± 70.5	N/A	N/A
	Danofloxacin	<MQL	<MQL	372.3 ± 414.1	N/A	<MQL
	Oxytetracycline	<MQL	<MQL	N/A	N/A	N/A
	Chloramphenicol	<MQL	157.9 ± 23.3	<MQL	<MQL	377.6 ± 46.3
	Penicillin G	N/A	<MQL	<MQL	N/A	N/A
	Penicillin V	<MQL	<MQL	<MQL	<MQL	N/A
	Erythromycin	2148.2 ± 168.5	6503.8 ± 619.1	13,591.5 ± 2445.9	N/A	N/A
	Prulifloxacin	N/A	<MQL	N/A	N/A	N/A
	Norfloxacin	N/A	N/A	N/A	N/A	N/A
Antifungal	Griseofulvin	<MQL	<MQL	<MQL	1.4 ± N/A	<MQL

Table 3 (continued)

Class of analyte	Compound	Surface water (ng L^{-1})	Effluent (ng L^{-1})	Influent (ng L^{-1})	SPM (ng L^{-1})	Digested solids (ng g^{-1})
	Ketoconazole	46.5 ± 6.1	64.3 ± 5.2	131.0 ± 40.9	171.6 ± 48.3	929.3 ± 212.1
Hypertension	Valsartan	<MQL	236.9 ± 23.0	854.9 ± 306.9	N/A	N/A
	Irbesartan	111.4 ± 14.6	287.9 ± 30.0	380.7 ± 73.3	N/A	N/A
	Lisinopril	<MQL	93.9 ± 16.1	890.5 ± 273.9	12.3 ± 2.7	<MQL
NSAIDs	Ketoprofen	<MQL	<MQL	<MQL	<MQL	<MQL
	Ibuprofen	60.5 ± 21.3	1029.2 ± 357.7	18,840.6 ± 5449.2	42.8 ± 15.9	231.8 ± 39.9
	Naproxen	234.9 ± 47.7	1452.5 ± 213.7	15,238.5 ± 3111.2	51.7 ± 17.9	108.4 ± 5.3
	Diclofenac	88.2 ± 7.3	345.6 ± 52.7	916.2 ± 367.3	<MQL	26.2 ± 3.4
	Acetaminophen	193.0 ± 49.7	1840.7 ± 449.3	393,559.0 ± 100,020.4	28.1 ± 23.7	<MQL
Lipid regulator	Bezafibrate	103.6 ± 15.8	591.7 ± 76.7	2038.3 ± 468.2	2.8 ± 1.2	7.5 ± 0.7
	Atorvastatin	44.4 ± 8.1	172.9 ± 27.0	1338.2 ± 456.4	N/A	N/A
Antihyperlipidaemic	Gemfibrozil	N/A	<MQL	N/A	N/A	849.6 ± 183.8
Antihyperintensive	Candesartan Cilexetil	N/A	N/A	N/A	N/A	N/A
Antihistamine	Fexofenadine	209.1 ± 31.9	732.6 ± 103.2	1012.1 ± 689.0	N/A	N/A
	Cetirizine	239.6 ± 35.8	1051.1 ± 154.5	1088.4 ± 106.6	N/A	N/A
GUD/ED	Sildenafil	2.2 ± 1.3	25.5 ± 12.4	29.0 ± 41.6	2.6 ± 2.9	20.8 ± 1.0
Diabetes	Metformin	3607.1 ± 413.6	14,050.8 ± 897.2	117,927.4 ± 24,688.7	N/A	N/A
	Gliclazide	47.3 ± 7.6	84.3 ± 7.5	136.4 ± 41.6	N/A	N/A
	Sitagliptin	142.5 ± 13.5	441.4 ± 67.2	524.7 ± 76.8	11.2 ± 2.9	27.6 ± 2.1
Cough suppressant	Pholcodine	<MQL	<MQL	<MQL	<MQL	<MQL
Beta blocker	Atenolol	53.9 ± 10.6	328.9 ± 31.4	2581.7 ± 584.4	23.3 ± 9.6	<MQL
	Metoprolol	<MQL	13.3 ± 2.9	32.1 ± 9.3	<MQL	<MQL
	Propranolol	20.6 ± 3.5	106.6 ± 6.1	182.3 ± 30.9	21.5 ± 6.4	198.5 ± 22.9
	Bisoprolol	<MQL	14.7 ± N/A	30.6 ± 29.3	<MQL	<MQL
H_2 receptor agonist	Ranitidine	148.2 ± 31.8	1163.0 ± 98.1	1545.0 ± 277.6	<MQL	<MQL
	Cimetidine	<MQL	<MQL	170.3 ± 64.4	N/A	N/A
X-ray contrast media	Iopromide	<MQL	<MQL	<MQL	N/A	N/A
Various	Buprenorphine	6.3 ± 0.2	5.7 ± N/A	60.8 ± 37.7	0.6 ± 0.2	13.2 ± 4.5
Drug precursor	Ephedrine/pseudoephedrine	25.6 ± 2.0	115.5 ± 18.6	655.5 ± 248.6	4.5 ± 1.3	<MQL
	Norephedrine	<MQL	<MQL	<MQL	<MQL	<MQL
Anticancer	Azathioprine	<MQL	<MQL	<MQL	N/A	N/A
	Methotrexate	<MQL	<MQL	<MQL	<MQL	<MQL
	Ifosfamide	<MQL	<MQL	<MQL	N/A	N/A
	Tamoxifen	<MQL	<MQL	<MQL	<MQL	<MQL

Table 3 (continued)

Class of analyte	Compound	Surface water (ng L^{-1})	Effluent (ng L^{-1})	Influent (ng L^{-1})	SPM (ng L^{-1})	Digested solids (ng g^{-1})
	Imatinib	38.3 ± 1.3	143.3 ± 39.6	88.8 ± 14.4	47.6 ± 17.1	123.0 ± 40.3
	Capecitabine	<MQL	<MQL	7.3 ± 2.8	<MQL	<MQL
	Bicalutamide	59.4 ± 1.1	115.6 ± 6.7	154.3 ± 10.0	7.5 ± 2.0	50.0 ± 11.6
Anaesthetic and metabolite	Ketamine	11.8 ± 2.7	75.4 ± 11.3	166.2 ± 65.6	0.4 ± 0.2	2.8 ± 0.5
	Norketamine	<MQL	5.7 ± 2.8	12.2 ± 7.0	<MQL	0.8 ± 0.2
Antidepressants and metabolites	Venlafaxine	85.5 ± 13.0	509.5 ± 135.8	462.4 ± 137.3	8.6 ± 2.0	126.3 ± 26.0
	Desmethylvenlafaxine	229.2 ± 23.9	981.0 ± 128.6	733.9 ± 129.3	2.9 ± 1.1	22.4 ± 1.8
	Fluoxetine	1.2 ± 0.1	46.2 ± 4.0	53.2 ± 7.1	43.7 ± 32.1	193.7 ± 33.2
	Norfluoxetine	<MQL	<MQL	31.8 ± 6.5	9.6 ± 2.2	91.4 ± 26.5
	Sertraline	<MQL	13.1 ± 1.7	46.3 ± 7.0	114.1 ± 34.8	565.2 ± 71.3
	Mirtazapine	4.2 ± 1.6	41.3 ± 2.4	78.2 ± 13.1	6.9 ± 2.3	69.1 ± 8.7
	Citalopram	<MQL	321.9 ± 19.9	557.5 ± 109.9	87.5 ± 25.7	782.9 ± 75.2
	Desmethylcitalopram	12.0 ± 4.5	119.7 ± 10.7	216.2 ± 59.1	31.7 ± 8.8	295.7 ± 62.6
	Paroxetine	<MQL	<MQL	N/A	1.8 ± N/A	3.2 ± 2.9
	Duloxetine	<MQL	<MQL	N/A	<MQL	17.5 ± 5.8
	Amitriptyline	10.3 ± 3.2	53.3 ± 6.9	159.4 ± 25.2	115.6 ± 26.3	471.9 ± 44.1
	Nortriptyline	7.8 ± 4.0	30.6 ± 3.0	9.6 ± 4.2	9.9 ± 3.0	63.2 ± 15.2
	Norsertraline	N/A	N/A	157.8 ± N/A	133.8 ± 37.8	N/A
Anti-epileptic	Carbamazepine	160.4 ± 16.8	626.8 ± 60.5	521.2 ± 115.3	7.2 ± 2.6	118.7 ± 11.5
	Carbamazepine-10, 11-epoxide	30.9 ± 4.2	138.2 ± 33.3	98.6 ± 29.9	N/A	N/A
	10,11-Dihydro-10-hydroxycarbamazepine	5.7 ± 2.1	43.2 ± 13.1	145.1 ± 55.1	<MQL	<MQL
Calcium channel blocker	Diltiazem	4.4 ± 2.0	59.5 ± 10.3	246.2 ± 55.5	N/A	N/A
	Verapamil	<MQL	<MQL	<MQL	5.8 ± 2.5	51.1 ± 6.1
Hypnotic	Temazepam	<MQL	21.5 ± 13.9	11.1 ± 7.4	<MQL	<MQL
	Oxazepam	2.0 ± 0.6	17.3 ± 4.1	16.6 ± 3.9	N/A	<MQL
	Diazepam	6.2 ± 0.8	11.6 ± 1.2	<MQL	0.9 ± 0.2	4.2 ± 0.4
Antipsychotic	Quetiapine	<MQL	<MQL	63.0 ± 31.4	2.9 ± 1.6	17.3 ± 4.7
	Risperidone	1.2 ± 1.4	2.6 ± 0.8	1.0 ± N/A	0.1 ± 0.1	<MQL
Dementia	Donepezil	<MQL	<MQL	N/A	0.9 ± 0.3	8.3 ± 0.2
	Memantine	<MQL	<MQL	<MQL	<MQL	<MQL
Human indicators	Creatinine	<MQL	<MQL	1,341,104.8 ± 314,821.7	N/A	N/A
	Nicotine	35.9 ± 16.1	183.9 ± 36.8	3461.2 ± 688.7	49.4 ± 26.3	227.1 ± 42.7
	Caffeine	426.5 ± 91.2	2938.9 ± 401.5	118,596.4 ± 24,610.9	N/A	N/A

Table 3 (continued)

Class of analyte	Compound	Surface water (ng L^{-1})	Effluent (ng L^{-1})	Influent (ng L^{-1})	SPM (ng L^{-1})	Digested solids (ng g^{-1})
	Cotinine	37.8 ± 5.7	214.5 ± 28.6	3028.3 ± 729.6	9.9 ± 2.9	40.1 ± 4.9
	1,7-Dimethylxanthine	923.5 ± 208.7	8352.1 ± 1091.1	153,776.3 ± 36,949.3	N/A	N/A
Analgesics and metabolites	Morphine	<MQL	141.7 ± 18.0	1366.1 ± 276.4	10.1 ± 3.8	63.2 ± 43.3
	Dihydromorphine	<MQL	<MQL	133.6 ± 32.7	<MQL	<MQL
	Normorphine	<MQL	<MQL	186.3 ± 50.7	<MQL	<MQL
	Methadone	3.0 ± 0.8	22.1 ± 1.5	41.6 ± 7.1	1.6 ± 0.5	12.9 ± 1.8
	EDDP	10.0 ± 1.5	50.3 ± 3.0	64.8 ± 12.9	11.0 ± 5.1	38.3 ± 22.3
	Codeine	85.6 ± 18.3	577.9 ± 58.6	2570.4 ± 555.0	23.3 ± 5.9	45.0 ± 42.5
	Norcodeine	<MQL	<MQL	181.0 ± 30.9	<MQL	<MQL
	Dihydrocodeine	22.1 ± 4.6	154.0 ± 19.3	445.2 ± 119.4	3.4 ± 0.9	24.8 ± 3.8
	Tramadol	321.7 ± 27.7	1273.7 ± 166.3	1247.5 ± 308.1	3.7 ± 1.0	34.5 ± 7.7
	N-Desmethyltramadol	255.3 ± 24.7	975.5 ± 118.3	1076.5 ± 355.9	1.5 ± 0.5	17.1 ± 5.6
	O-Desmethyltramadol	297.0 ± 32.9	1300.4 ± 200.0	861.6 ± 133.9	N/A	N/A
Stimulants and metabolites	Amphetamine	<MQL	<MQL	478.8 ± 135.7	<MQL	<MQL
	Methamphetamine	<MQL	9.1 ± 0.8	13.9 ± 3.7	<MQL	<MQL
	MDMA	4.3 ± 1.9	59.4 ± 33.3	183.2 ± 127.7	1.4 ± 0.9	6.1 ± 1.0
	MDA	10.0 ± 0.9	30.1 ± 7.6	45.5 ± N/A	N/A	N/A
	Cocaine	4.9 ± 1.6	47.1 ± 11.7	754.6 ± 295.3	6.9 ± 3.2	<MQL
	Benzoylecgonine	45.5 ± 9.9	291.2 ± 95.7	2078.4 ± 925.3	1.9 ± 1.1	<MQL
	Anhydroecgonine methyl ester	<MQL	<MQL	<MQL	N/A	N/A
	Cocaethylene	<MQL	3.6 ± 1.1	29.3 ± 18.5	0.4 ± 0.3	<MQL
	Mephedrone	<MQL	<MQL	<MQL	<MQL	<MQL
	MDPV	<MQL	<MQL	<MQL	<MQL	<MQL
Opioid and metabolite	Heroin	<MQL	<MQL	<MQL	<MQL	<MQL
	6-Acetylmorphine	<MQL	<MQL	<MQL	N/A	N/A
Pesticides, fungicides and herbicides	Thiamethoxam	<MQL	<MQL	<MQL	<MQL	<MQL
	Imidacloprid	53.0 ± 18.5	346.6 ± 133.7	339.2 ± 178.8	<MQL	N/A
	Clothianidin	<MQL	<MQL	<MQL	1.8 ± 0.8	N/A
	Metazachlor	4.5 ± N/A	<MQL	<MQL	4.2 ± 4.1	<MQL
	Terbuthylazine	<MQL	<MQL	<MQL	<MQL	<MQL
	Methiocarb	<MQL	3.0 ± N/A	3.7 ± 0.6	0.8 ± 0.6	1.7 ± 0.0
	Dichlofluanid	N/A	N/A	N/A	N/A	N/A
	Flufenacet	24.0 ± 2.4	61.8 ± 3.5	57.1 ± 12.9	4.1 ± 0.7	14.7 ± 0.5

Table 3 (continued)

Class of analyte	Compound	Surface water (ng L^{-1})	Effluent (ng L^{-1})	Influent (ng L^{-1})	SPM (ng L^{-1})	Digested solids (ng g^{-1})
Veterinary pharmaceuticals	Oxadiazon	16.9 ± 3.4	29.8 ± 3.4	<MQL	9.3 ± 0.4	<MQL
	Chlorpyrifos	<MQL	<MQL	N/A	N/A	93.8 ± N/A
	Triallate	<MQL	<MQL	<MQL	N/A	N/A
	Tylosin	<MQL	<MQL	<MQL	N/A	N/A
	Sulfapyridine	128.7 ± 19.6	576.0 ± 121.2	1339.5 ± 330.3	N/A	N/A
	Sarafloxacin	<MQL	<MQL	N/A	N/A	N/A
	Ceftiofur	31.2 ± N/A	216.1 ± 77.2	451.8 ± 129.2	N/A	N/A
	Diazinon	6.1 ± 0.3	12.0 ± 0.3	17.5 ± 0.4	11.9 ± 3.9	15.2 ± 3.6

used, (b) limited sources or databases used in studying ecotoxicity data and (c) varying criteria in accepting ecotoxicity study results. This has highlighted the need to harmonise these methods to ensure PNECs are calculated consistently, to provide comparable comparisons between studies and to be clearer of the risks CECs pose to the environment.

Regarding digested solids, the concentrations are in ng g^{-1} of solid material, which cannot be directly compared to the concentrations of the other matrices, as it is a combination of sludge from various parts of the wastewater treatment process. However, it is an important consideration as a source of CECs in the environment, due to subsequent direct application to the land in agricultural practices. Of the 96 analytes that can be quantified with this method, 55% were found at this site. Antidepressants are high in concentration, with average concentrations of five of the analytes between 126.3 and 782.9 ng g^{-1}. Of the more industrial CECs, methylparaben and bisphenol A are present in solids at high levels. In particular, bisphenol A has been quantified at levels exceeding 4000 ng g^{-1} at this site. Gemfibrozil, an antihyperlipidaemic, although not found in other matrices at this site, was quantified in digested solids at a concentration of 849.6 ± 183.8 ng g^{-1}. This suggests accumulation of gemfibrozil in other sections of the WwTW that were not analysed. Due to the low MQL, the lack of incoming concentration during the sampling period suggests occasional loads high in gemfibrozil before the study.

Conclusions

This work presents a validated multi-residue method for the analysis of 195 compounds in five matrices (3 liquid and 2 solid). These CECs cover a variety classes, both veterinary and human pharmaceuticals, industrial chemical, personal care products and pesticides. Application of the method to environmental matrices has shown that the method is appropriate for assessing treatment efficiency, partitioning to solids, and environmental concentrations. Discussion of the results has identified several key areas regarding environmental risk assessment, e.g. PNECs that need to be addressed; however, that is outside the scope of this paper. The achieved MDL and MQL concentrations appear low enough to be used to assess the environmental risk of these CECs. The results show a need for analysing both the liquid and solid phases within a WwTW; however, it also indicates a need to monitor all outgoing treated waste materials, e.g. effluent and digested sludge. This was due to the appearance of gemfibrozil, which was not present at quantifiable levels in any other matrix at this site. Overall, this method is suitable to be used in catchment-based exposure-driven studies to further increase knowledge of the contribution of CECs by WwTWs to the environment and the risk they pose.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Kathryn Proctor is currently finishing her PhD, joint funded by NERC and Wessex Water, at the University of Bath. She has been working for several years on developing and applying methods for exposure and effects-driven analysis (UPLC-MS/MS, toxicity assays) of chemicals of emerging concern in the environment, both in Bath and abroad in Argentina as part of INTERWASTE (Synergising International Research Studies into the Environmental Fate and Behaviour of Toxic Organic Chemicals in the Waste Stream).

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Appendix 6B: Statement of Authorship

This declaration concerns the article entitled:								
Micropollutant fluxes in urban environment – a catchment perspective								
Publication status (tick one)								
Draft manuscript	**X**	**Submitted**		**In review**		**Accepted**		**Published**	**X**
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Copyright status (tick the appropriate statement)								
I hold the copyright for this material				Copyright is retained by the publisher, but I have been given permission to replicate the material here	X			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	Formulation of ideas: The candidate was considerable contributor to ideas expressed. Design of methodology: The candidate was considerable contributor to the design of the sampling campaign. Experimental work: The candidate was one of the major contributors in the sample preparation of solid and liquids samples and their analysis. Presentation of data in journal format: The candidate was the major contributor for the data analysis, interpretation, and presentation of the data in this paper.							
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.							
Signed	[redacted]			**Date**	11/12/2020			

Chapter 3

Micropollutant fluxes in urban environment – a catchment perspective

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Abstract

This study provided a holistic understanding of the sources, fate and behaviour of 142 compounds of emerging concern (CECs) throughout a river catchment impacted by 5 major urban areas. Of the incoming 169.3 kg d^{-1} of CECs entering the WwTWs, 167.9 kg d^{-1} was present in the liquid phase of influent and 1.4 kg d^{-1} was present in the solid phase (solid particulate matter, SPM). Analysis of SPM was important to determine accurate loads of incoming antidepressants and antifungal compounds, which are primarily found in the solid phase. Furthermore, these classes and the plasticiser, bisphenol A (BPA) were the highest contributors to CEC load in digested solids. Population normalised loads showed little variation across the catchment at 154 ± 12 mg d^{-1} $inhabitant^{-1}$ indicating that population size is the main driver of CECs in the studied catchment. Across the catchment 154.6 kg d^{-1} were removed from the liquid phase during treatment processes. CECs discharged into surface waters from individual WwTWs contributed between 0.19 kg d^{-1} at WwTW A to 7.3 kg d^{-1} at WwTW E, which correlated strongly with the respective contributing populations. Spatial and temporal variations of individual CECs and their respective classes were found in WwTW influent (both solid ($influent_{SPM}$) and liquid phases ($influent_{AQ}$)) throughout the catchment, showing that different urban areas impact the catchment in different ways, with key variables being lifestyle, use of over-the-counter pharmaceuticals and industrial activity. Understanding of both spatial and temporal variation of CECs at the catchment level helped to identify possible instances of direct disposal, as in the case of carbamazepine. Analysis of surface waters throughout the catchment showed increasing mass loads of CECs from upstream of WwTW A to downstream at WwTW D, showing clear individual contributions from WwTWs. Many CECs were ubiquitous throughout the river water in the catchment. Daily loads ranged from 0.005 g d^{-1} (ketamine, WwTW A) up to 1890.3 g d^{-1} (metformin, WwTW C) for the 84/138 CECs that were

detected downstream of the WwTWs. For metformin this represents the equivalent of ~1,890 tablets (1,000 mg per tablet) dissolved in the river water downstream of WwTW C.

Key words: micropollutants, catchment, pharmaceuticals, pesticides, endocrine disruptors, river, wastewater, solids

1. Introduction

Anthropogenic substances, such as pharmaceuticals, pesticides, plasticizers, UV filters, industrial chemicals etc., have been widely recognised to be entering the environment from a variety of sources. Many of these substances, particularly pharmaceuticals and personal care products ingredients, enter primarily via point sources such as wastewater treatment works (WwTWs), or for other classes such as veterinary pharmaceuticals and pesticides, as diffuse sources such as agriculture.

There are many studies that detail the presence of a range of compounds in a variety of matrices, however the majority of this existing work has been focused on one or two classes at a time, or a small number of compounds of emerging concern (CECs), primarily in aqueous matrices (Boogaerts et al., 2019; Loos et al., 2009; Mole and Brooks, 2019; Musolff et al., 2009; Petrie et al., 2014a). There is a broad range of existing data (Geissen et al., 2015; Petrie et al., 2014a; Sousa et al., 2018) due to the large number of potential substances, matrices, methods, and multiple lines of investigation that can be pursued. Much of this data cannot be compared directly due to the different methods utilised, as they have different quantification parameters. Even the sampling process can have a huge effect on how the results are interpreted, methodological details are often lacking (Ort, C., Lawrence, M. G., Rieckermann, J., and Joss, 2010; Ort et al., 2010).

Work investigating solid matrices such as solid particulate matter (SPM), activated and digested sludge, sediments and soils is less common, due to the difficulty of analysing such complicated matrices. Analysis of solid matrices alongside liquid matrices is critical for a better understanding of the fate and impact of many compounds (Langdon et al., 2012; Petrie et al., 2014a). Some CECs, such as antidepressants, are excreted in or adsorb to SPM before they reach the WwTWs, as well as being released during treatment (Baker and Kasprzyk-Hordern, 2011). The solids produced during WwTW processes, are treated to remove excess water and dangerous pathogens by a variety of processes. This digested sludge, usually termed ‘biosolids’ is often applied directly to soil as it is rich in nutrients suitable for crops (Kinney et al., 2006; Langdon et al., 2012), but these biosolids have been widely found to be a concentrated source of contaminants. Despite this the CEC content in biosolids is not monitored.

Despite the limitations of studies discussed above, they clearly show that a single wastewater or environmental sample can or has the potential to contain many different CECs from different classes. Furthermore, many studies have shown the products of metabolism, degradation and transformation of many of these CECs are/have the potential to also be present. Overall this leads to a very complex issue in understanding true exposure levels in the environment and the potential risk they may pose.

Identification of mixtures of co-occurring, high risk CECs, or priority mixtures, is one of the challenges in water quality monitoring (Altenburger et al., 2015). To gain further understanding of these mixtures, their consistency/fluxes within the environment will allow a better understanding of the environmental risk posed by these CECs. Understanding the fluxes of these mixtures will allow the potential changes in risks to be anticipated, potentially leading to optimised treatment and mitigation of risk to the environment. Currently, further work is required to investigate the composition of the mixture in samples from a range of matrices. This will not only require analysis of the mixtures present, but it will provide insight into spatial and temporal trends, between matrices and across a catchment.

The aim of the paper is to investigate the changes in micropollutant load throughout a river catchment system in the South-West of the UK, to gain further information on their sources, fate and behaviour. This was achieved by undertaking a comprehensive investigation of 142 CECs, previously prioritised and analytical method validated (Proctor et al., 2019), at five strategic WwTWs representing >75% of the catchment population. At each WwTW, influent (both liquid and solid phases) and effluent wastewater, digested solids, and upstream and downstream river water were monitored for 7 consecutive days. Five aspects were considered: 1) spatial and temporal variations in the influent, 2) partitioning between aqueous ($influent_{AQ}$) and solid phases ($influent_{SPM}$) in the influent, 3) percentage removal of CECs from the liquid phase, 4) mixture profiles of CECs in all matrices, and 5) spatial trends in river water composition throughout the catchment. This provides a high resolution and more holistic view of the distribution of these CECs throughout the catchment.

Table 1 *Classes of CECs with names of analytes per matrix, total number of analytes in each matrix in first row.*

Class	Compound	River	Effluent	Influent	SPM	Dig. Solids
UV Filter	Benzophenone-1	4	4	4	3	3
	Benzophenone-2					
	Benzophenone-3					
	Benzophenone-4					
Parabens	Methylparaben	4	4	4	4	4
	Ethylparaben					
	Propylparaben					
	Butylparaben					
Plasticizer	Bisphenol-A	1	1	1	1	1
Steroid Estrogens	E1	3	3	3	2	2
	E2					
	EE2					
Antibiotics and Antibacterial	Sulfasalazine	19	20	19	7	7
	Clarithromycin					
	Azithromycin					
	Trimethoprim					
	Sulfamethoxazole					
	Triclosan					
	Amoxicillin					
	Metronidazole					
	Sulfadiazine					
	Cefalexin					
	Ofloxacin					
	Ciprofloxacin					
	Tetracycline					
	Danofloxacin					
	Oxytetracycline					
	Chloramphenicol					
	Penicillin G					
	Penicillin V					
	Erythromycin					
	Prulifloxacin					
	Norfloxacin					
Antifungal	Griseofulvin	2	2	2	2	2
	Ketoconazole					
Hypertension	Valsartan	3	3	3	1	1
	Irbesartan					
	Lisinopril					
NSAIDs	Ketoprofen	5	5	5	5	5
	Ibuprofen					
	Naproxen					
	Diclofenac					
	Acetaminophen					
Lipid regulator	Bezafibrate	2	2	2	1	1
	Atorvostatin					
Anti-hyperlipidemic	Gemfibrozil	1	1	1	0	1
Anti-hyperintensive	Candesartan Cilexetil	1	0	0	0	0
Antihistamine	Fexofenadine	2	2	2	0	0
	Cetirizine					
GUD/ED	Sildenafil	1	1	1	1	1
Antidiabetics	Metformin	3	3	3	1	1
	Gliclazide					
	Sitagliptin					
Cough suppressant	Pholcodine	1	1	1	1	1
Beta-blocker	Atenolol	4	4	4	4	4
	Metoprolol					
	Propranolol					
	Bisoprolol					
H2 receptor agonist	Ranitidine	2	2	2	1	1
	Cimetidine					
X-ray contrast media	Iopromide	1	1	1	1	1
Various	Buprenorphine	1	1	1	1	1
Drug precursor	Ephedrine/pseudoephedrine	2	2	2	2	2
	Norephedrine					
Anti-cancer	Azathioprine	7	7	7	5	5
	Methotrexate					
	Ifosfamide					
	Tamoxifen					
	Imatinib					
	Capecitabine					
	Bicalutamide					

Class	Compound	River	Effluent	Influent	SPM	Dig. Solids
Anaesthetic and metabolite	Ketamine	2	2	2	2	2
	Norketamine					
Anti-depressants	Venlafaxine	12	12	13	13	12
	Desvenlafaxine					
	Fluoxetine					
	Norfluoxetine					
	Sertraline					
	Mirtazapine					
	Citalopram					
	Desmethylcitalopram					
	Paroxetine					
	Duloxetine					
	Amitriptyline					
	Nortriptyline					
	Norsertraline					
Anti-epileptic	Carbamazepine	3	3	3	2	2
	Carbamazepine10,11-epoxide					
	10,11-Dihydro-10-hydroxycarbamazepine					
Calcium-channel blocker	Diltiazem	2	2	2	1	1
	Verapamil					
Hypnotic	Temazepam	3	3	3	2	2
	Oxazepam					
	Diazepam					
Anti-psychotic	Quetiapine	2	2	2	2	2
	Risperidone					
Dementia	Donepezil	2	2	2	2	2
	Memantine					
Creatinine	Creatinine	5	5	5	2	2
Lifestyle Chemicals	Nicotine					
	Caffeine					
	Cotinine					
	1,7 dimethylxantine					
Analgaesics and Metabolites	Morphine	11	11	11	10	10
	Dihydromorphine					
	Normorphine					
	Methadone					
	EDDP					
	Codeine					
	Norcodeine					
	Dihydrocodeine					
	Tramadol					
	N-desmethyltramadol					
	O-desmethyltramadol					
Stimulants and metabolites	Amphetamine	10	10	10	8	8
	Methamphetamine					
	MDMA					
	MDA					
	Cocaine					
	Benzoylecgonine					
	Anhydroecgonine methylester					
	Cocaethylene					
	Mephedrone					
	MDPV					
Opioid and metabolite	Heroin	2	2	2	1	1
	6-acetylmorphine					
Pesticides, fungicides and herbicides	Thiamethoxam	10	10	10	8	7
	Imidacloprid					
	Clothiniadin					
	Metazachlor					
	Terbuthylazine					
	Methiocarb					
	Dichlofluanid					
	Flufenacet					
	Oxadiazon					
	Chlorpyrifos					
	Triallate					
Veterinary Pharma	Tylosin	5	5	5	1	1
	Sulfapyridine					
	Sarafloxacin					
	Ceftiofur					
	Diazinon					

2. Materials and methods

2.1. Materials

All materials used in the investigation are detailed in the Supporting information (SI), Section S1. The analytical standards were of the highest purity possible and purchased from Sigma Aldrich, LGC standards or Toronto Research Chemicals (TRC). The solvents used were of HPLC grade. All glassware was silonised to prevent losses of analytes to the untreated glassware. The classes covered by this study are shown in Table 1. Due to the wide range of CECs and complex matrices, not all CECs could be validated for every matrix. Table 1 shows the CECs which are present in each class (green box) and which are validated for each matrix (Proctor et al., 2019).

2.2. Sampling methods and location

Samples were collected at each of the five WwTWs (A-E) for 7 consecutive days between June and October 2015. The five WwTWs utilise a range of treatment technology and different sized populations (Table 2). Sampling was carried out using volume proportional sampling for influent wastewater, time-proportional for effluent and grab sampling for river water upstream and downstream of the effluent discharge point (sample point distance from discharge point is in Table 2). Digested sludge was collected, via grab sampling, on three consecutive days from WwTW B and WwTW E. Further detail and discussion on the methods and location used can be found in the SI: Section S1, 2.1 and 2.2.

2.3. Sample preparation and analysis

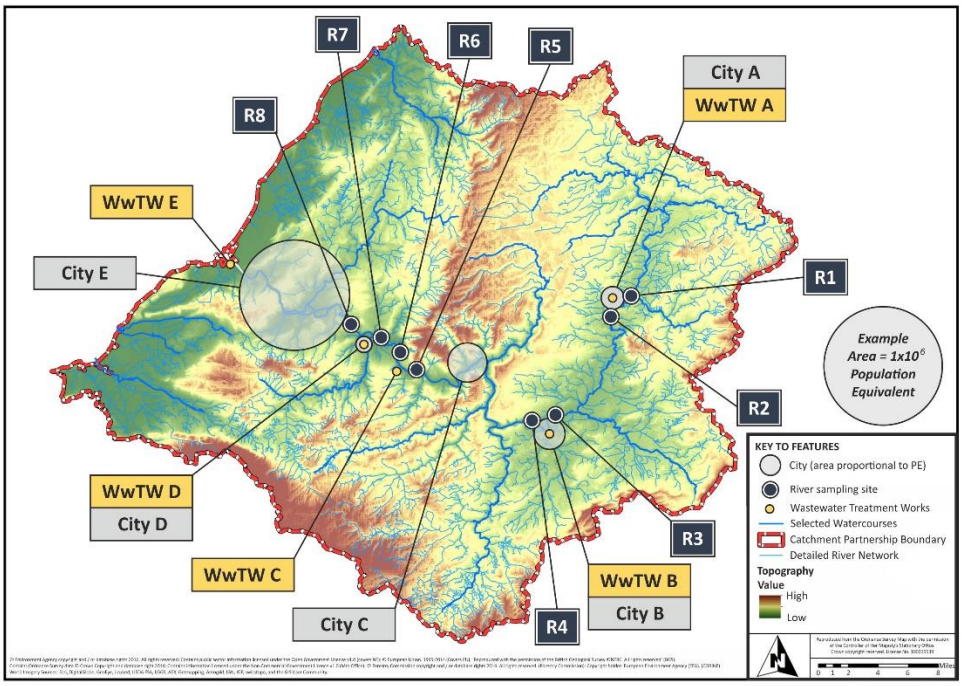
Liquid samples were spiked with internal standards and analytes extracted by solid phase extraction (SPE) using OASIS HLB cartridges before analysis with ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) (Waters). The solid samples were frozen, freeze-dried, homogenised, weighed and spiked with internal standard before undergoing microwave assisted extraction (MAE) followed by SPE with OASIS MCX cartridges. Further detail and discussion on the methods used can be found in the SI, Section S1, 2.3.

2.4. Quality control

To ensure the quality of generated data, spiked quality control samples were analysed for both liquid and solid matrices. All samples were spiked with internal standards to compensate for matrix suppression effects, as well as any losses of analyte during sample preparation. All sample analysis was performed in duplicate.

A further element of quality control was considered with regards to river water sampling. To ensure downstream river waters were completely mixed with effluent, mass balances were estimated for

Table 2 *Site information of studied STWs and corresponding river locations*



Site	SWRT (h)	WwTW secondary process[b]	SRT (d)	HRT[a] (h)	Media type	Config.	Pop. served	Indust. %	Mean flow (m^3 d^{-1})	River sampling, distance to discharge point (km)		Effluent-river mass balance[c] (%)
										Up	Down	
A	<0.5-4	AS	19	46.2	n/a	Carbonaceous & nitrifying	37,000	0.4 %	8,242 ± 3,085	0.5	n/a[d]	n/a[d]
B	<0.5-4	TF	n/a	24.5	Stone	Carbonaceous & nitrifying	67,870	30.0 %	11,202 ± 3,202	0.5	0.5	102
C	<0.5-9	TF	n/a	13.9	Stone – limestone	Carbonaceous & nitrifying	105,847	1.2 %	24,875 ± 2,167	2	2	111
D	<0.5-2	TF	n/a	17.6	Stone – blast furnace slag	Carbonaceous & nitrifying	17,638	0.1 %	2,924 ± 199	1	1	97
E	<1-24	90 % SBR 10 % AS	4 8	10.9 25.8	n/a	Carbonaceous	909,617	23.9 %	153,061 ± 12,245	n/a[e]	n/a	n/a

Key: WwTW, wastewater treatment process; SWRT, sewer residence time; SRT, solids retention time; HRT, hydraulic retention time; p, 'pulses' or toilet flushes, AS, activated sludge; TF, trickling filter; SBR, sequencing batch reactor

[a] Under typical summer flows

[b] All WwTWs utilised primary sedimentation dosed with ferric sulfate for phosphorus removal and all processes used conventional sedimentation following secondary treatment except SBRs which decanted following settling *in-situ*

[c] Mass balances were calculated according to: $Mass\ balance\ (\%) = \frac{Downstream}{Upstream + Effluent} \times 100$ where *Downstream* is the load of carbamazepine in river water downstream of the effluent discharge point (g d^{-1}), *Upstream* is the load of carbamazepine in river water upstream of the effluent discharge point (g d^{-1}) and *Effluent* is the load of carbamazepine in effluent (g d^{-1})

[d] Mass balance at site E was > 400 % demonstrating complete mixing of effluent and river water was not achieved at the sampling point due to restricted access to river. Therefore mass loads in river water downstream of the discharge point was calculated by adding effluent loads with river water loads upstream of the discharge point. This assumes complete mixing without any micropollutants losses. Micropollutant concentrations in downstream river water were then estimated using river flow data.

[e] Effluent discharged into estuary

carbamazepine (e.g. Equation 1). Carbamazepine was selected due to its resistance to biological degradation and photodegradation, which is expected to be negligible over the short distances between sampling points (Heberer, 2002). Further discussion of this can be found in the SI, Section S1, 2.2.1 and Section S2 and results can be found in Table 2.

3. Results and discussion

The discussion of results in this paper is primarily in loads, i.e. g d^{-1}, as it allows direct comparison between different matrices and sites. Number of CECs per class (c) and number of samples with measurable concentration in each matrix (n) are discussed for some CECs within the text and can be found for all classes in Table 1. General chemical information and physicochemical parameters of the CECs of interest is gathered in Table S8. Further information is available in the SI.

3.1. Solid-liquid phase distribution of CECs within communal discharges

Overall, 112 of the 138 CECs quantifiable in $influent_{AQ}$ were detected at least once during the study entering the five WwTWs. 74 of the 96 micropollutants were quantified in $influent_{SPM}$. 39 of which were found in all $influent_{AQ}$ and $influent_{SPM}$ samples with classes ranging from antidepressants, analgesics and their metabolites to illicit stimulants e.g. cocaine and industrial chemicals such as parabens, the plasticiser BPA and the UV filter, benzophenone-1.

The chemical content of each phase of influent is distinctly different (Figure 1 and 2). With lifestyle chemicals, such as caffeine, nicotine and their metabolites, NSAIDs (and acetaminophen) and antidiabetics, predominantly found in the aqueous phase (99.4 %, 99.8 % and 96.2 % of the total load of each chemical present in the aqueous phase, on average across the catchment) and making up the majority of the incoming wastewater. Whilst $influent_{SPM}$, is primarily made up of the plasticiser, BPA (69.6 %), antidepressants (12.9 %) and antifungals (4.1 %). The latter two of which in particular show high levels of sorption to the solid phase over the aqueous, 36.3 % (including metabolites) and 55.4 % respectively.

Much of the differences between the $influent_{AQ}$ and $influent_{SPM}$, is of course likely due to the physicochemical characteristics of these compounds such as their log K_{ow}, and water solubility. For example, the NSAIDs: ibuprofen, naproxen and acetaminophen have log K_{ow} values of 3.79, 3.10 and 0.29 respectively and water solubility of 41.1, 145, and 30400 mg L^{-1} and all are primarily found in $influent_{AQ}$ (0.3%, 0.3% and 0.01% of the total load of each compound). These levels of partitioning are far lower than previously reported by Samaras et al. (Samaras et al., 2013), however similar phase distribution was shown by Petrie et al. for crude wastewater (Petrie et al., 2014b). This may be due to differences between WwTWs sewer retention time, as well as physicochemical properties of the matrix

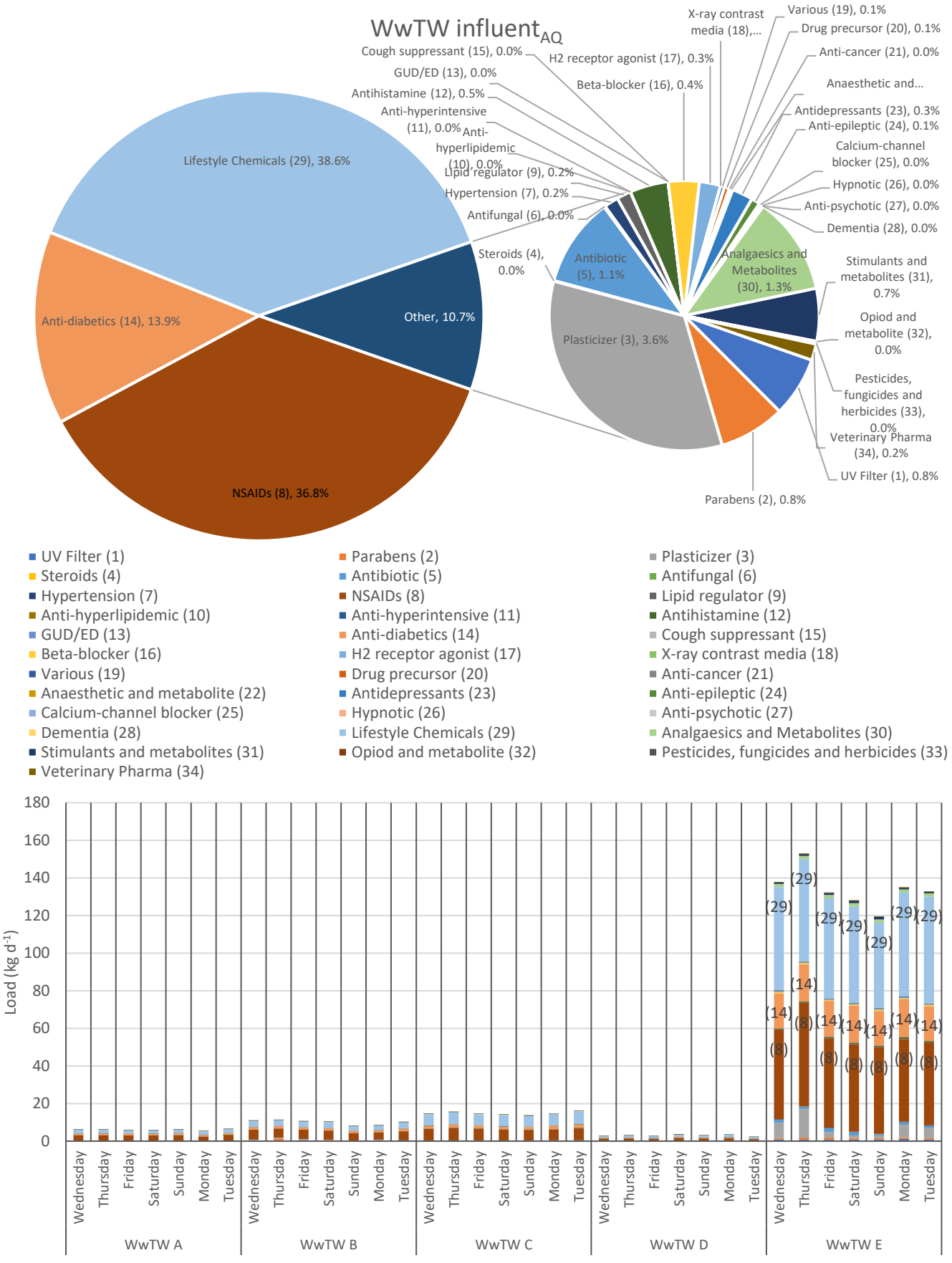


Figure 1 *Weekly percentage of total loads in influent$_{AQ}$ of the entire catchment as a pie chart of classes, with chart showing spatial and temporal trends. Note: creatinine is not included.0.0% shows negligible to no contribution to the total. Numbers in brackets indicate numbers assigned for identification in small figures and table*

(e.g. pH). Despite these low levels of partitioning, ibuprofen, naproxen and acetaminophen are in the top 20 CEC contributors (16, 12, 11 respectively) to total influent$_{SPM}$ load in this study with daily loads of 8.6, 10.1, 11.8 g d^{-1} (or 6.0 – 9.1 mg d^{-1} 1000 inh^{-1} (ibuprofen), 5.7 – 12.8 mg d^{-1} 1000 inh^{-1} (naproxen) and 4.6 – 13.2 mg d^{-1} 1000 inh^{-1} (acetaminophen) if considering population normalised loads). Within influent$_{SPM}$ these three painkillers show similar loads, however with the influent$_{AQ}$ phase, acetaminophen has a much higher normalised load; 44.8 – 77.0 g d^{-1} 1000 inh^{-1} (18.0 % daily variation across the catchment). Whilst, loads for naproxen and ibuprofen were much lower with 3.1 ± 0.6 g d^{-1} 1,000 inh^{-1} and 2.7 ± 0.7 g d^{-1} 1,000 inh^{-1} respectively. These pharmaceuticals are commonly found in the influent$_{AQ}$ of many WwTWs across the globe, due to their high usage and availability without a prescription (Sousa et al., 2018). This is despite low excretion rates due to the extensive metabolism of these NSAIDs (Luo et al., 2014). These results are similar to those found by Mendoza et al., where ibuprofen, naproxen and acetaminophen were found to be the most abundant pharmaceuticals of the study (Mendoza et al., 2015; Paíga et al., 2019). Diclofenac and ketoprofen, which are not so readily available over the counter in the UK, present much lower loads (131.2 ± 37.9 mg d^{-1} 1,000 inh^{-1} (n = 35) and 8.7 ± 17.5 mg d^{-1} 1,000 inh^{-1}, (n = 7) respectively) in influent$_{AQ}$ and less frequently in the case of ketoprofen, which only appears at WwTW E. Despite their worldwide use and abundance, their presence in influent$_{SPM}$ is often overlooked.

As previously mentioned, antidepressants and antifungals are two classes for which a high proportion of the total incoming load can be found within influent$_{SPM}$ (36.3% and 55.4 % respectively). Antidepressants (no. of analytes = 13) contribute 12.9 % to the total influent$_{SPM}$ load and antifungals (no. of analytes = 2) contribute 4.1 % (Figure 2). All antidepressants and metabolites in this study, apart from paroxetine (3.95 log K_{ow}, 35.3 mg L^{-1} water solubility) and duloxetine (4.68 log K_{ow}, 13.0 mg L^{-1}) can be found in influent$_{SPM}$. With log K_{ow} of the parent compound ranging from 3.28 (venlafaxine) to 5.29 (sertraline), the percentage of the total load of each compound found in influent$_{SPM}$ is between 2.9 % (venlafaxine) to 67.0 % (sertraline). These results are not unusual and similar data has been obtained from wastewater samples collected in a week long study in the Czech Republic (CR) and over a yearlong study in the UK by Baker et al., (Baker et al., 2012; Baker and Kasprzyk-Hordern, 2013). The presence of antifungals, on the other hand, is primarily due to ketoconazole (log K_{ow} 4.45). This CEC is primarily found in the influent$_{SPM}$, with 55.8 % of the load in this phase. This result is comparable to a study by Peng et al. (Peng et al., 2012), who also found ketoconazole primarily in influent$_{SPM}$. In that study, other azoles were also analysed, such as fluconazole, clotrimazole, miconazole, and econazole, all of which were found in influent$_{SPM}$ only and not influent$_{AQ}$, showing that this may be a key matrix to investigate for this class and a wider range of antifungals should be considered in future.

Overall lifestyle chemicals, have the highest contribution to this catchment with 38.6% of the load, furthermore the daily variation of this load, normalised by population for these compounds, over the

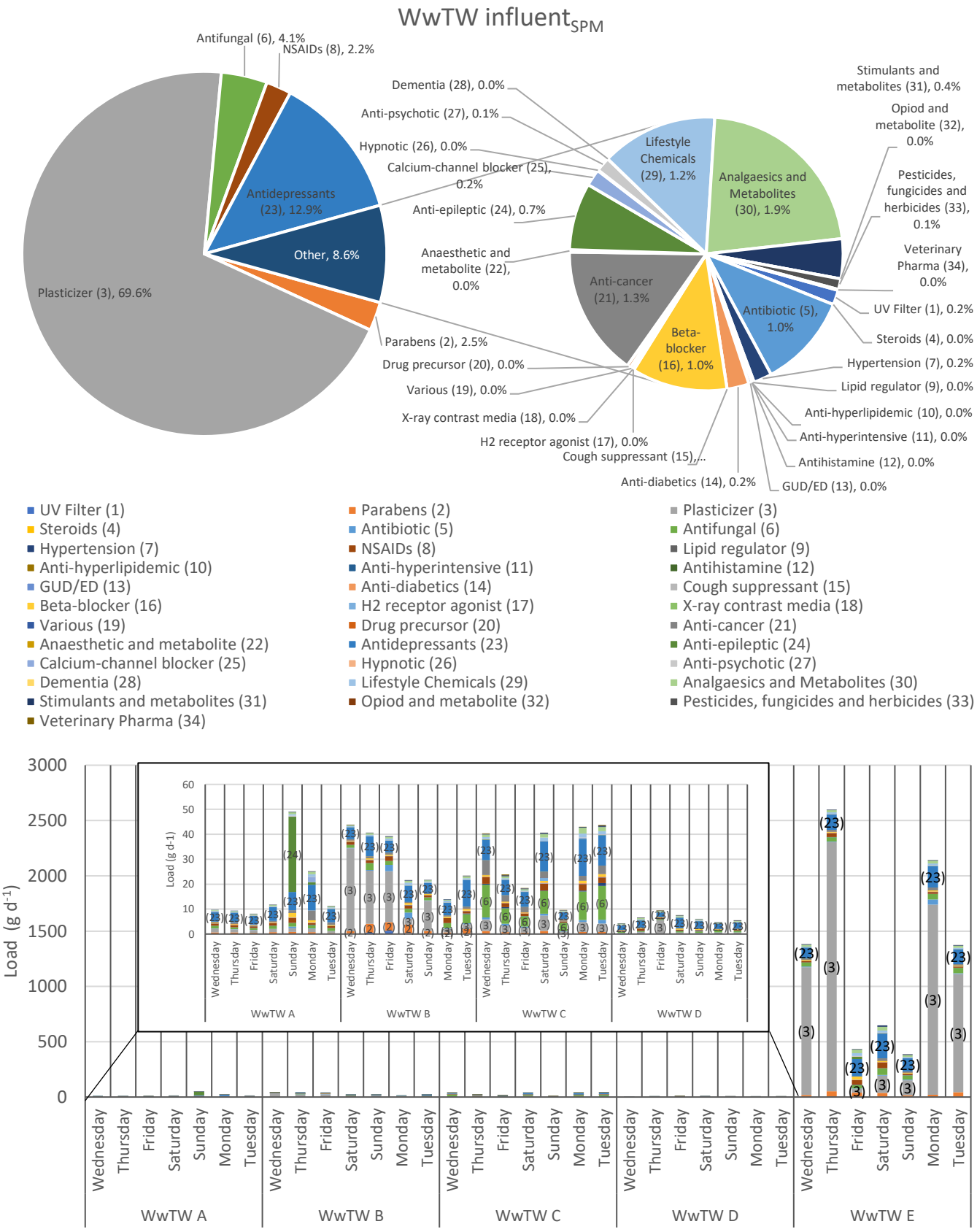


Figure 2 *Weekly percentage of total loads in influent$_{SPM}$ of the entire catchment as a pie chart of classes, with chart showing spatial and temporal trends. Note: creatinine is not included. 0.0% shows negligible to no contribution to the total. Numbers in brackets indicate numbers assigned for identification in small figures and table*

seven days of sampling at each of the five sites (n = 35), shows caffeine has one of the lowest daily load variations (23.1 %) of most compounds in this study. The other lifestyle chemicals show more variation; 1,7-dimethylxanthine (26.5 %), nicotine (42.5 %) and its metabolite cotinine (26.2 %) and creatinine (52.1 %). This can provide some insight into the patterns of people's lifestyle habits across a catchment. As an example, wastewater-based epidemiology was applied to caffeine and its metabolite 1,7-dimethylxanthine (methodological details can be found in the SI, Section S3, 1.2.1) to understand usage patterns across the catchment. Overall it was found the loads present suggest an intake of 26 – 57 mg of caffeine per person per day, which is in line with a cup of coffee of a few cups of black tea per day (de Mejia and Ramirez-Mares, 2014; Wishart et al., 2018).

The loads calculated in influentSPM represent a large proportion of antidepressants and antifungals but also for other individual compounds; the anti-cancer drug imatinib (39.9 % partitioning to influent$_{SPM}$) and the anti-psychotic risperidone (87.7 % partitioning to influent$_{SPM}$). For some CECs, such as verapamil, thiamethoxam, oxadiazon, methiocarb and donepezil, influent$_{SPM}$ represents all of the total load for these compounds and is therefore the primary route of entry of these CECs to the environment, which may have gone undetected in studies which focus only on the influent$_{AQ}$. SPM matrix is therefore key to understanding the fate of these classes and CECs.

Various factors are considered important in the consideration of partitioning between liquid and solid phases, these include, water solubility, log K_{ow}, partition coefficient (K_d), log D_{ow}, as well as a compounds polarity and structure. In theory, the likelihood of a compound to sorb to the solid phase increases with log K_{ow} (Hyland et al., 2012). In this study, when considering the classes individually, there is some correlation between these factors, however, considering the full range of CECs the simplistic model of 'the higher the log K_{ow}, the more partitioning to solids' cannot be easily applied. Further work is needed to understand this behaviour.

3.2. Spatial and temporal CEC trends in WwTWs

3.2.1. Overall spatial and temporal trends of CEC loads

The spatial and temporal trends (Figure 1 and 2) of the overall load, in both influent$_{AQ}$ and influent$_{SPM,}$ shows that similar chemical speciation between these two matrices is observed across all WwTWs within this catchment, with the loads in influent$_{AQ}$ being primarily driven by population size (Table S3 and Figure 1). The five WwTWs ranged in size from 18,274 to 867,244 population equivalents. The incoming flow ratio of residential population to commercial/trade also varied from site to site, which is displayed as a percentage of the total population equivalents in Table 2. However, influent$_{SPM}$, (Table S4 and Figure 2) shows there is far more temporal and spatial variation than appears in the influent$_{AQ.}$

3.2.1.1. Industrial chemicals

Figure 1 shows a correlation can be seen between higher industrial contributions to wastewater seen at WwTW B (30.0%) and E (23.9%),the total weekly load of BPA, UV filters and parabens and particularly the weekly influent$_{SPM}$ load e.g. 92.8 g week^{-1} (B) to 6522 g week^{-1} (E) (BPA), 2.9 g week^{-1} (B) to 10.3 g week^{-1} (E) (UV filters), and 14.8 g week^{-1} (B) to 218.5 g week^{-1} (E) (parabens) compared with 7.5 g week^{-1} (D) to 23.6 g week^{-1} (C), 0.1 g week^{-1} (D) to 0.6 g week^{-1} (C), 1.2 g week^{-1} (D) to 5.0 g week^{-1} (C) respectively at the other WwTWs. This can also be seen in the population normalised loads (Figure 1 and 2), although the correlation is far clearer in the influent$_{SPM}$, than the influent$_{AQ}$. BPA, in particular, contributes 45.4 % (WwTW B) to 72.8 % (WwTW E) to the total load of influent$_{SPM}$ throughout the campaign. This equates to total population equivalent normalised loads of 29.5 – 694.3 mg d^{-1} 1,000 inh^{-1} and 40.6 – 2827 mg d^{-1} 1,000 inh^{-1} for WwTWs B and E, respectively. When comparing these results to the 7.0 %, 10.8 % and 16.8 % (partitioning to influent$_{SPM}$) or 6.7 mg d^{-1} 1,000 inh^{-1} (minimum at C) to 307.2 mg d^{-1} 1,000 inh^{-1} (maximum at D), it is a considerable portion. Furthermore, clear temporal trends can also be seen for BPA in both phases (Figure S3), showing increasing levels throughout the working week, reducing to lower levels over the weekend. The presence of BPA in domestic wastewater has previously been linked to leaching from plastics, such as pipes or drinking bottles which would account for the low level loads commonly seen (Flint et al., 2012; Petrie et al., 2019; Rubin, 2011). The increase levels from industrial waste may be linked to the production of epoxy resins, polycarbonate plastics and thermoprinting paper, however it has not been linked to a specific trade within this catchment at this time. The presence and trends of this compound in this catchment is described in more detail by Petrie et al. and Lopardo et al. (Lopardo et al., 2019; Petrie et al., 2019).

The personal care product ingredient methylparaben, also shows specific industrial spatial and temporal trends. It is present at a constant level across the week at WwTWs A, C and D, with normalised loads in influent$_{AQ}$ ranging from 564.6 – 976.1 mg d^{-1} 1,000 inh^{-1}. It is often found in personal care products such as shampoos and shower gels. Therefore, for this CEC, a consistent level across the week is expected. However, at WwTWs with higher industrial input e.g. WwTW B and E, the trends seen in influent$_{AQ}$ show significant increase of methylparaben on certain days of the week, which may be as a result of relevant industrial processes, such as toiletry manufacture, which is known to be present in the area. These trends can be seen in both influent$_{AQ}$ and influent$_{SPM}$ (FigureS3), as levels increase from across the working week and decrease over the weekend (up to 16,242 mg d^{-1} 1000 inh^{-1} on Thursday to 681.0 mg d^{-1} 1,000 inh^{-1} on Sunday in influent$_{AQ}$ and up to 48.2 mg d^{-1} 1,000 inh^{-1} on Thursday to 8.5 mg d^{-1} 1,000 inh^{-1} on Sunday in influent$_{SPM}$ at WwTW B, whereas for WwTW E the trends are strongest in the influent$_{SPM}$ with trends increasing up to 41.9 mg d^{-1} 1,000 inh^{-1} on Friday to 15.5 mg d^{-1} 1,000 inh^{-1} on Sunday). The influence of industrial activity on the highly variable loads of these chemicals, may have a significant environmental impact, if they are not effectively removed.

3.2.1.2. Illicit drugs

Spatial trends were also observed for some illicit stimulants, demonstrating variation in the usage behaviour throughout the catchment area. It was postulated that those areas with the greater population size and night life (WwTWs C and E) would see the greater loads of illicit stimulants (e.g. MDMA, cocaine, amphetamine and mephedrone) due to recreational usage. Cocaine, amphetamine and MDMA followed this trend. For example, at WwTWs C and E, total MDMA loads (sum of both $influent_{AQ}$ and $influent_{SPM}$) were found up to 120.8 and 157.1 mg d^{-1} 1,000 inh^{-1} respectively (Tables S3 and S4). At the remaining sites, maximum loads were found in the range 33.3 – 79.9 mg d^{-1} 1,000 inh^{-1}. Previous studies have found cocaine, amphetamine and MDMA use to be greater in large urban populations than in smaller more rural locations (Lai et al., 2016; Nefau et al., 2013). In contrast, mephedrone loads were highest at WwTW D which treats wastewater from the smallest population size (18,274 inhabitants). Total influent loads ranged between 13.1 and 38.9 mg d^{-1} 1,000 inh^{-1} in comparison to loads of 3.8 to 8.5 mg d^{-1} 1,000 inh^{-1} at WwTW C and 7.2 to 20.5 mg d^{-1} 1,000 inh^{-1} at WwTW E (Table S3 and S4). Mephedrone was not detected in wastewater at WwTWs A and B.

The weekly trends for stimulants are also very pronounced (Figure S2). There was an increasing weekend load of not only MDMA and cocaine but also their metabolites: MDA (MDMA), benzoylecgonine (cocaine) and cocaethylene (combination of cocaine and alcohol), but not anhydroecgonine methylester (metabolite from smoking crack cocaine). This shows increased usage of both MDMA and cocaine throughout the catchment during the weekend, though this is less pronounced in areas that are less populated, more rural and with less night life. These trends have previously been seen on numerous occasions across the world (US, (Gushgari et al., 2018), Czech Republic (Baker et al., 2012), England and Europe (Castrignanò et al., 2018b), China (Zhang et al., 2019). The trends, shown in Figure S3, can also been seen in $influent_{SPM}$ for both cocaine, benzoylecgonine and MDMA, despite there being proportionately less load present in $influent_{SPM}$, 1.4 %, 0.1 %, and 0.9 % respectively. Interestingly, a spike in load is observed on one day for $influent_{SPM,}$ rather than over the entire weekend for $influent_{AQ}$.

3.2.1.3. Pharmaceuticals linked to hospital effluent

Total population normalised loads of the analgesic morphine were greater at WwTWs C and E. With ranges between 377.5 to 607.6 mg d^{-1} 1,000 inh^{-1} at WwTW C and 372.2 to 443.1 mg d^{-1} 1,000 inh^{-1} at WwTW E, compared to the other sites which ranged between 184.5 mg d^{-1} 1,000 inh^{-1} at WwTW A to 284.9 mg d^{-1} 1,000 inh^{-1}, also at WwTW A (the ranges of the remaining two WwTWs are quite similar and fall within this range (Tables S3 and S4). Higher morphine loads at WwTWs C and E can be attributed to hospitals within their catchment areas, similar to a study conducted in Portugal, which found that 51 % of the total analgesic load in municipal wastewater was from hospitals (Santos et al.,

2013). However, within this catchment a more detailed investigation is required to confirm the contribution of hospital wastewater. Furthermore, the anti-cancer drug ifosfamide was only detected in wastewater at WwTWs C and E (Table S3 and Table S4). Although ifosfamide is not directly linked to hospital wastewater, as it can be excreted from the homes of patients receiving chemotherapy, it was not detected at WwTWs which did not receive hospital wastewater.

3.2.1.4. Lifestyle chemicals and pharmaceuticals

Many CECs, such as lifestyle chemicals and some NSAIDs, which are freely available without prescription and used widely, show little variation between sites across the catchment e.g. caffeine, with average loads of 23,826 ± 5,498 mg d^{-1} 1,000 inh^{-1}, showing 23.1% daily variation across the catchment, acetaminophen, with 58,374 ± 10,494 mg d^{-1} 1,000 inh^{-1} and 18.0%, and ibuprofen with 3,092 ± 629 mg d^{-1} 1,000 inh^{-1} and 20.4%.

This trend continues with many pharmaceuticals which are prescribed widely for chronic conditions e.g. the anti-diabetic, metformin, (daily variation across the catchment = 21.5 %, with average total influent load of 20,260 ± 4,357 mg d^{-1} 1,000 inh^{-1}), analgesic for moderate pain, tramadol, (17.4 %, 241.3 ± 42.1 mg d^{-1} 1,000 inh^{-1}), and the antidepressants, citalopram (14.5 %, 108.0 ± 15.6 mg d^{-1} 1,000 inh^{-1}) and amitriptyline (20.5 %, 53.9 ± 11.1 mg d^{-1} 1,000 inh^{-1}). Interestingly, compounds in the same class, which appear at much lower loads, show more spatial variation and minimal temporal variation e.g. the anti-diabetic, sitagliptin (35.7 %, 70.2 ± 25.0 mg d^{-1} 1,000 inh^{-1}), and the antidepressant, fluoxetine (41.0 %, 20.7 ± 8.5 mg d^{-1} 1,000 inh^{-1}). This may be a sign of variation in prescribing behaviour of healthcare professionals (Rowlingson et al., 2013), spatial variation in the prevalence of relevant conditions, or it may be due to differences in the stability of the pharmaceutical within the sewer and the difference in sewer residence time to the site. This has been found to be an issue with illicit drug monitoring and other pharmaceuticals have shown the potential to degrade within sewers (Gao et al., 2017; Jelic et al., 2015; McCall et al., 2016). Further investigation is required to provide a more detailed assessment.

Antibiotics and antibacterial compounds (c = 19), only contribute a small proportion, 1.1 %, to the total $influent_{AQ}$ load, and $influent_{SPM}$ load, 1.0% (c =7). Several of these CECs, such as sulfasalazine, clarithromycin, azithromycin, trimethoprim, sulfamethoxazole and triclosan were found in all $influent_{AQ}$ samples at all WwTWs (with the exception of azithromycin, which was missing from one sample at WwTW A), but showed highly variable population normalised loads (Table S3). Within $influent_{SPM}$, only trimethoprim was found in all samples, ranging from 1.4 mg d^{-1} 1,000 inh^{-1} at WwTW B to 13.7 mg d^{-1} 1,000 inh^{-1} at WwTW C. Few other antibiotics were found in SPM, only sulfadiazine was found with some regularity and only at WwTW B (100% of samples at population normalised loads between 0.9 to 2.2 mg d^{-1} 1,000 inh^{-1}). Unfortunately, this method was unable to quantify

fluoroquinolones in this matrix, a class of antibiotics known for their ability to partition to the solid phase (Castrignanò et al., 2018a; Martín et al., 2015; Petrie et al., 2014b), therefore the antibiotic load of this matrix is likely to be underestimated for these compounds. Despite this it is clear that these compounds are widely used (from influent$_{AQ}$ results) and two, azithromycin and clarithromycin, have been placed on the WFD Watch List as substances of potential environmental concern (Carvalho et al., 2015).

Ciprofloxacin and erythromycin are also present on this list and yet within this catchment they are detected less frequently within the influent$_{AQ}$ (n = 7 and 21), though their loads, when found, are significant (ciprofloxacin 15.8 ± 10.6 g d-1 at WwTW A only, and erythromycin is found at levels between 9.0 ± 1.9 g d-1 at WwTW D to 189.6 ± 13.6 g d-1 at WwTW E). Other antibiotics, such as metronidazole, sulfadiazine, cefalexin, ofloxacin, tetracycline, danofloxacin, and chloramphenicol are found sporadically in the influent$_{AQ}$ throughout the catchment, often at lower loads than the other antibiotics. Their sporadic presence may be due to limited use. Further consideration of prescription levels will provide a clearer understanding, but this is outside the scope of this paper.

Trends of the population normalised loads for antibiotic and antibacterial compounds show some variation between WwTWs and between individual compounds. For example, WwTW B shows the highest population normalised loads for sulfasalazine (93.1 ± 28.5 mg d^{-1} 1,000 inh^{-1} compared to 45.0 ± 15.1 mg d^{-1} 1,000 inh^{-1} at WwTW A which has the lowest), azithromycin (135.7 ± 70.4 mg d^{-1} 1,000 inh^{-1} compared to 21.9 ± 15.5 mg d^{-1} 1,000 inh^{-1} at the lowest at WwTW A), and triclosan (405.5 ± 181.1 mg d^{-1} 1,000 inh^{-1} compared to 154.1 ± 10.2 mg d^{-1} 1,000 inh^{-1} at the lowest at WwTW C). However, WwTW B also has the lowest levels for other antibiotics such as clarithromycin (209.8 ± 49.4 mg d^{-1} 1,000 inh^{-1} compared to 369 ± 86.6 mg d^{-1} 1,000 inh^{-1} at WwTW D), trimethoprim (99.0 ± 7.8 mg d^{-1} 1,000 inh^{-1} compared to 247.1 ± 21.5 mg d^{-1} 1,000 inh^{-1} at WwTW C), and the second lowest for sulfamethoxazole levels at 18.8 ± 6.7 mg d^{-1} 1,000 inh^{-1}, which is less than 20 % of the highest levels (100.5 ± 6.6 mg d^{-1} 1,000 inh^{-1} WwTW E). This variation may be due to differences in the prescription practices, which could be influenced by variable uptake of prescription advice from the Government as part of the UK Five Year Antimicrobial Resistance Strategy (Department of Health & and Department for Environment Food and Rural Affairs United Kingdom, 2013).

Some CECs, particularly pharmaceuticals, that are regularly and widely used by the population, show no temporal trends throughout the week. This is to be expected, as those pharmaceuticals that are sporadically but widely used, such as NSAIDs and painkillers e.g. acetaminophen and ibuprofen (Figure S2), will show only small variations in load. Other pharmaceuticals, such as antibiotics, are used in treating specific conditions and often require courses of several days, but may be prescribed less often, so are used less widely. Antibiotics, such as sulfamethoxazole and trimethoprim which are often

prescribed together (as co-trimoxazole) as a long administration course (14 - 21 days), show a steady trend across the week. Other antibiotics, with typically shorter courses, such as azithromycin, clarithromycin, metronidazole and ciprofloxacin, show more variation across the week. To see trends of these compounds, longer term studies are required to cover time periods encompassing seasons or even years, such as those performed in CR, Greece, Spain, and New Zealand (Golovko et al., 2014; Kumar et al., 2019; Mastroianni et al., 2017; Papageorgiou et al., 2016). This would be particularly useful for antibiotics as it will indicate whether reducing prescription reduces the influent load and any seasonal trends may indicate incorrect prescribing practices (from prescriptions of antibiotics for flu during winter months for which it is not effective) (Coutu et al., 2013; Golovko et al., 2014).

3.2.1.5. Veterinary pharma and pesticides

Surprisingly, the veterinary antibiotic, sulfapyridine, is present at population normalised loads, for total influent, ranging from 205.4 ± 23.8 mg d^{-1} inh^{-1} to 299.8 ± 25.8 mg d^{-1} inh^{-1} and shows little daily variation (17.8 %) across the sampling campaign. It has been found previously at low level in $influent_{AQ}$ and its presence has been linked to human use (Ebele et al., 2017; Golovko et al., 2014; Paíga et al., 2016; Wilkinson et al., 2017) as well as veterinary use (Sarmah et al., 2006). However, this antibiotic is no longer prescribed or advised for use by humans in the UK, as it is of critical importance for use with food producing animals, but it is also produced during the human metabolism of sulfasalazine (European Medicines Agency, 2019; Kasprzyk-Hordern et al., 2008; Peppercorn, 1984; Wishart et al., 2018). In this study, it is thought this metabolism of sulfasalazine may be the main source contributing to sulfapyridine's consistent presence across the catchment. This can also be seen in the similarity of their temporal and spatial trends. It is thought that if the main contributing factor was due to usage on livestock, its presence would not be consistent across the catchment, as large variances between rural areas (WwTW B) and highly urban areas (WwTW E) would be expected. Furthermore, the similarity in temporal and spatial trends with sulfasalazine would be very unlikely. Sarafloxacin and diazinon were the only other veterinary pharmaceuticals found, with sarafloxacin only found at in one $influent_{AQ}$ sample at WwTW D at 5.7 mg d^{-1} 1000 inh^{-1} and diazinon found across the catchment in 80% of the $influent_{AQ}$ samples and 22.9% of the $influent_{SPM}$ samples at total influent loads ranging from 0.6 mg d^{-1}1000 inh^{-1} (WwTW C) to 85.5 mg d^{-1} 1000 inh^{-1} (WwTW E). Interestingly, diazinon is primarily found in influent from the larger WwTWs serving the two major cities. This is it perhaps an indication of a larger numbers of pets relative to inhabitants in these areas compared to more rural areas, or a higher prevalence in the use of deworming medication for which it is primarily used. Overall, veterinary pharmaceuticals and pesticides represent a small proportion, < 0.5% of the total influent chemical load, of the CECs analysed.

3.2.1.6. Anticipated and accidental micropollutant fluxes

Considering the temporal and spatial distribution of CECs across the catchment allows a better understanding over the micropollutant mixtures and fluxes of load that are experienced by the WwTWs, allowing for pattern to emerge regarding human behaviour, degradation and seasonal changes in larger studies. This will allow the loads and fluxes to be anticipated allowing optimisation of treatment technologies for better removal of these contaminants. However, studying the trends in this work anomalies can be detected.

Figure 2 shows a significantly higher proportion of the total load of influent$_{SPM}$ is due to antifungals, specifically ketoconazole, as griseofulvin was not found at this site. Ketoconazole was found in all influent$_{SPM}$ samples at all sites, showing its frequent and widespread use. At WwTW C however, the normalised loads were on average 79.2 ± 35.7 mg d^{-1} 1,000 inh^{-1} compared to the 27.5 to 50.2 mg d^{-1} 1,000 inh^{-1} at the other sites. The high standard deviation seen at WwTW C compared to the other sites may be more indicative of incorrect usage, incidental release or direct disposal rather than difference in prescription.

A similar situation is seen at WwTW A, as anti-epileptics represent a far higher proportion of influent$_{SPM}$ (25.4 %, Figure 2). This is entirely due to the presence of the parent compound as the metabolite 10,11-dihydro-10-hydroxycarbamazepine was not detected in influent$_{SPM}$ and the other metabolite, carbamazepine-10,11-epoxide could not be analysed in influent$_{SPM}$. The normalised loads of carbamazepine at WwTWs B-E were in the range of <MQL (1 sample at WwTW D) to 5.3 ± 8.0 mg d^{-1} 1,000 inh^{-1}, whilst at WwTW A they were 119.3 ± 287.4 mg d^{-1} 1,000 inh^{-1}. This standard deviation indicates a very skewed distribution of carbamazepine load at WwTW A, which is not consistent for a pharmaceutical used solely to treat chronic conditions. This is likely a further example of incorrect usage or direct disposal of unused carbamazepine. To gain further understanding of this distribution, the temporal trends were considered.

For ketoconazole, with a normalised load of 79.2 ± 35.7 mg d^{-1} 1,000 inh^{-1} at WwTW C, it shows high daily variation through the week (45.0%) with high loads seen on Monday-Wednesday and lower throughout the rest of the week. A similar trend is also seen at WwTW B with the highest loads on Tuesday and Wednesday and a daily variation of 44.1 %, the other sites have daily variation of 21.5 – 29.7 %. From further research, this is likely due to the primary mode of administration of this pharmaceutical in the form of a medicated shampoo (based on prescription data from this catchment), which is applied one to two times a week for the prevention or treatment of seborrheic dermatitis and dandruff (20 mg g^{-1}) and is available both over the counter and with a prescription (National Health Service Business Services Authority, 2019; Wishart et al., 2018).

Carbamazepine shows a significant increase in influent$_{SPM}$ load on Sunday at WwTW A, which is not seen in the metabolites. In influent$_{AQ}$, the carbamazepine load increases by >300 %, from the average load of 4.3 g d^{-1} to 12.7 g d^{-1}. Carbamazepine has previously shown no measurable degradation under typical sewer conditions (O'Brien et al., 2017), therefore the levels seen are likely unchanged from entering the sewer. Within the catchment of this WwTW, this pharmaceutical is mainly administered in tablet form as 100, 200, or 400 mg (National Health Service Business Services Authority, 2019; Wishart et al., 2018). Therefore, this peak represents disposal of between 21 × 400 mg tablets or 84 × 100 mg tablets. In influent$_{SPM}$, the same trend can be seen, however it occurs to a greater magnitude (from the mean of the rest of the week: 0.2 g d^{-1} to 30.2 g d^{-1} on Sunday). When influent$_{SPM}$ is combined with influent to calculate a total load, the increase is from 4.5 g d^{-1} to 42.9 g d^{-1}, which suggests disposal between 96 × 400 mg tablets or 384 × 100 mg tablets. The percentage partitioning for this day was drastically altered from the 3.6 % average for the remainder of the week to the high value to 70 % on the day. This is perhaps indicative of the disposal of a highly concentrated solid load.

Fluoxetine disposal has been previously observed within this catchment, which was attributed to ~915 pills, as described by Petrie et al. (Petrie et al., 2016), adding to evidence which suggests direct disposal of pharmaceuticals is more common than previously thought. Within that study Petrie et al. proposed a framework to differentiate between normal, daily usage of these CECs and direct disposal of them in influent. It is likely that the use of 24-hour composites with a short period between subsample collection allowed these events to be captured. Currently, the effects of these unexpected spikes of CECs are unknown. The biological treatments at WwTWs will largely adapt to the everyday fluxes of CEC load, however, the sudden increase in CECs such as carbamazepine, ketoconazole or fluoxetine could potentially cause changes in the microbiology that reduce treatment efficiency. Furthermore, these events will likely lead to an increase in load and concentration leaving the works, which may cause a similar phenomenon with the environmental flora and fauna, as it is exposed to an acute impact of CEC load.

3.2.2. CEC removal from the liquid phase during WwTW treatment

The catchment-scale study enabled the performance of five WwTWs for the removal of micropollutants to be assessed under similar weather conditions (Table S9). Percentage removal (% removal) was calculated as described in Section S2, SI. To summarise, it is the percentage reduction in load of a CEC between liquid phases of influent (influent$_{AQ}$) and effluent. The process types monitored include two activated sludge treatments, conventional activated sludge (CAS) (WwTWs A and E), and sequencing batch reactors (SBRs) (WwTW E). Trickling (rotating biological) filters (TF) configured with different bed media were used at the remaining WwTWs (WwTW B, C and D). CAS is generally considered to achieve greater micropollutant removals than TFs from collated full-scale data (Baker and Kasprzyk-

Hordern, 2013; Kasprzyk-Hordern et al., 2009). This is considered to be as a result of longer hydraulic retention times (HRT) associated with CAS, enabling greater contact time for biodegradation. HRTs for this catchment can be found in Table 2. However, this study found this is not the case for all classes of CECs. Figure S1 shows average percentage removals ± relative standard deviation per site and overall removal in bar charts, the data for which can be found in Table S7. Figure 3 shows the removal data of selected classes of CECs across all WwTWs in the form of box plots.

The removal of lifestyle chemicals and creatinine were high, with creatinine removed at 99.6 ± 0.9 %, caffeine at 97.8 ± 1.8 %, nicotine at 96.6 ± 3.1 %, 1,7-dimethylxanthine at 95.6 ± 3.6 % and cotinine at 93.2 ± 5.7 %. The CAS and SBR WwTWs (WwTWs A, E) show better removals for caffeine and nicotine and significantly better removals for their metabolites. This is in line with removals seen at other sites in the UK with TFs and CAS in a study by Baker et al (Baker and Kasprzyk-Hordern, 2013).

This trend can be seen in the NSAIDs, where this pattern continues with acetaminophen (only slight improvement at WwTWs A, E due to such high removal 99.4 ± 0.7 %), ibuprofen (94.4 ± 5.3 %), and naproxen (83.0 ± 12.9 %). In contrast, diclofenac shows the best removal at sites with TFs (WwTWs B-D, removal range 29.0 – 64.5 %), and worst at WwTW E (-3.0 ± 10.7 %). Ketoprofen showed 11.4 ± 9.9 % removal at WwTW E but was not detected at the other sites and therefore removal cannot be determined. The trend seen for the other NSAIDs is consistent with those found by Martín et al. and Kasprzyk-Hordern et al., (Kasprzyk-Hordern et al., 2009; Martín et al., 2012).

The plasticiser, BPA (93.0 ± 3.6 %), and other industrial and personal care product ingredients, generally show high removal across the catchment with little variation between sites, such as the UV filters (benzophenone-1 with 96.6 ± 3.1 %, benzophenone-2 with 99.6 ± 0.8 %, benzophenone-3 with 91.7 ± 2.0 %, not benzophenone-4 with 32.6 ± 32.3 % removal however), and all parabens (methylparaben with 99.5 ± 0.3 %, ethylparaben with 99.8 ± 0.4 %, propylparaben with 99.2 ± 0.7 % and butylparaben with 100.0 ± 0.0 % removal). This is consistent with removals obtained for these CECs at sites with TFs and CAS treatment in Wales (Kasprzyk-Hordern et al., 2009).

Several antidepressants show low-medium level removal with little variation between TF WwTWs B-D, i.e. citalopram (average removals are between 17.3 to 20.5 %), amitriptyline (50.9 to 57.6 %) and sertraline (53.1 to 58.2 %), but show the medium to high levels of removal at CAS WwTW A (51.5 ± 19.4 %, 87.6 ± 10.2 %, and 54.4 ± 24.1 % for citalopram, amitriptyline and sertraline respectively). Kasprzyk-Hordern et al., found similar levels of removal for amitriptyline at both TFs and CAS sites (Kasprzyk-Hordern et al., 2009). Mirtazapine shows similar levels of removal for WwTWs A-C (22.0 ± 6.3 %) and had the highest levels of removal at WwTW D (39.8 ± 11.4 %). Venlafaxine saw negative removals at WwTWs A-C (-28.8 ± 14.5 %) and, similarly to mirtazapine, showed the highest levels of removal at WwTW D, 28.4 ± 23.6 %. Fluoxetine also shows negative removal at WwTW A-B (-53.8

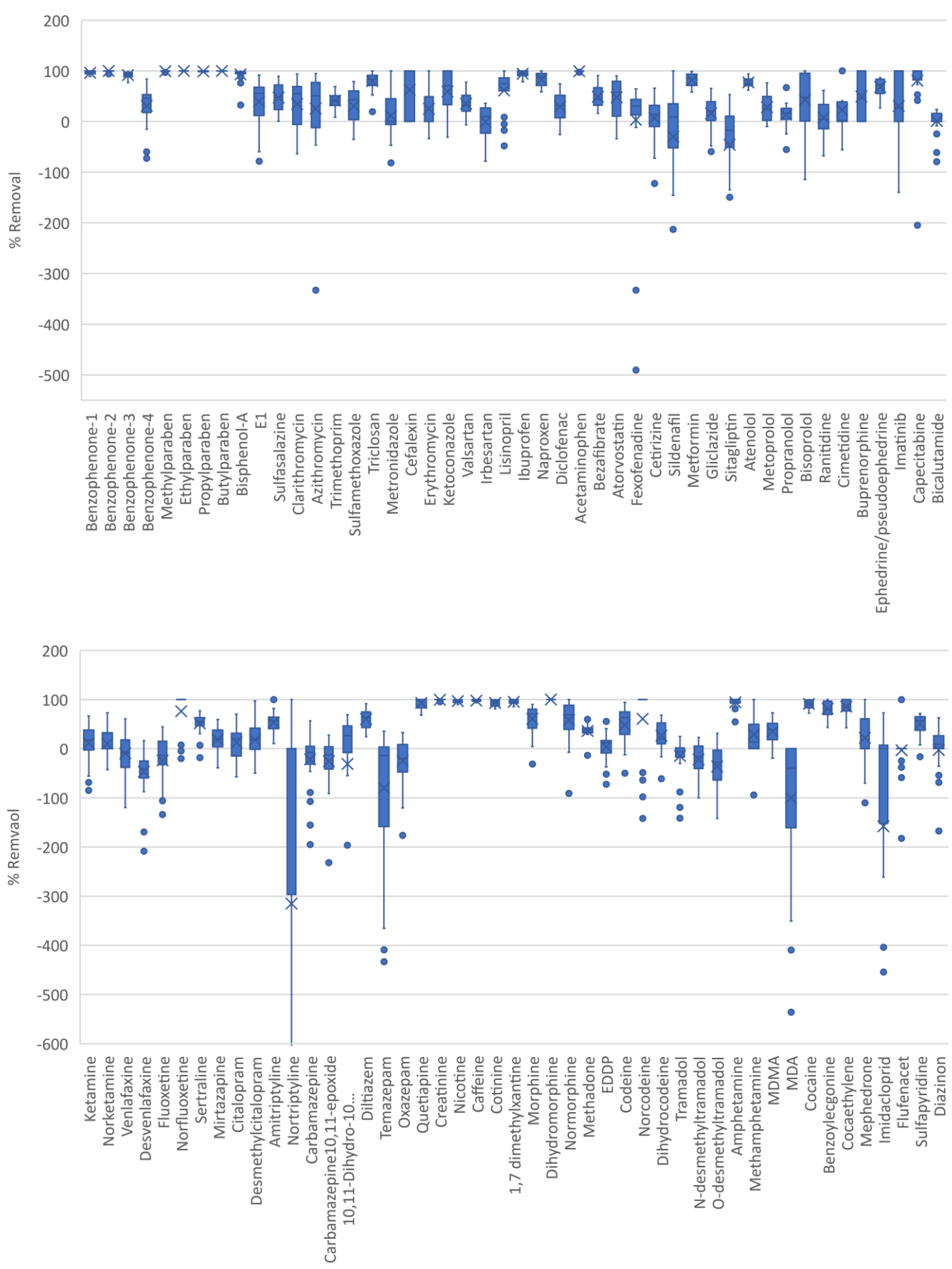


Figure 3 *Box plots showing removal of CECs from the liquid phase during WwTW treatment for each site and overall.*

to -27.4 %), WwTW C showed high highest levels of removal 32.7 ± 8.9 %, no overall removal at WwTW D. Both venlafaxine and fluoxetine have previously shown greater removal levels at both TF and CAS sites (Baker and Kasprzyk-Hordern, 2013; Verlicchi et al., 2012). WwTW E showed the worst removals for all antidepressants, ranging from -81.3 % for fluoxetine to 35.7 % for sertraline (except venlafaxine, which showed negligible removal at this site), this may be due to the short hydraulic residence time (HRT = 10.9 h) in the main treatment stream (90 % sequencing batch rectors) at this site. The antidepressant metabolites were either completely removed (norfluoxetine, norsertraline), similar to results found by Baker et al., and Comber et al., (Baker and Kasprzyk-Hordern, 2013; Comber et al., 2019), were removed similarly to the parent drug (desmethylcitalopram), or increased in load between influent$_{AQ}$ and effluent, likely due to degradation of the parent drug into the metabolite (nortriptyline, desmethylvenlafaxine), similar to what was found by Baker et al. and Paiga et al., (concentration based calculation of removal, rather than load) (Baker and Kasprzyk-Hordern, 2013; Paíga et al., 2019).

Carbamazepine and its metabolites, carbamazepine-10,11-epoxide and 10,11-dihydro-10-hydroxycarbamazepine, show increased levels between influent$_{AQ}$ and effluent at the CAS WwTW A. 10,11-dihydro-10-hydroxycarbamazepine forms O-glucuronides during human metabolism, which can be cleaved by β-glucuronidase, from faecal bacteria, leading to this increase (Ta et al., 1999). Carbamazepine and carbamazepine-10,11-epoxide, on the other hand, form N-glucuronides during human metabolism, which have shown they cannot be degraded by this enzyme but still show increased loads in effluent (Bahlmann et al., 2014).

The lack of degradation for tramadol in this study contrasts with the results found by Baker et al., for both TFs and CAS, however, it is comparable to removal levels found by Kasprzyk-Hordern et al., and Archer et al., (Archer et al., 2017; Baker and Kasprzyk-Hordern, 2013; Kasprzyk-Hordern et al., 2009). The O-desmethyltramadol metabolite can be further metabolised to form O-glucuronides (Wishart et al., 2018), which as previously discussed, are cleaved during biological treatment.

The high removal of the lifestyle chemicals, NSAIDs, parabens and plasticisers has led to a very different profile for treated wastewater compared to raw wastewater. This is observed in analgesics and metabolites, which represent a quarter of the total load after treatment. Anti-diabetics also show an increased proportion of the total load, due to relatively low removal at the WwTWs. Overall, antibiotics are poorly removed, < 50 %, although WwTWs A and E have higher levels of removal for sulfasalazine (73.7 ± 9.2 % WwTW A and 71.8 ± 3.2 % at WwTW E) and clarithromycin (83.0 ± 9.8 % WwTW A and 64.3 ± 7.3 % WwTW E). WwTW E removed 74.2 ± 7.3 % and 68.7 ± 6.1 % of azithromycin and sulfamethoxazole respectively, but A has very poor removal for these compounds. WwTWs using biological activated sludge have previously shown reasonable removal for these compounds, similar to what was seen at WwTW E in this study (Golovko et al., 2014). Furthermore, it shows that long term

seasonal changes may have further effects on removal that are not seen in this study, but which should be taken into account for the wider picture.

In summary, although, previously CAS was considered a better micropollutant removal process than TFs, this considered a smaller range of compounds (Baker and Kasprzyk-Hordern, 2013; Kasprzyk-Hordern et al., 2009). The larger range of compounds considered in this study shows this is not so clear cut and there is great variation between classes, as well as CECs within the classes. In the next section overall mass balance is taken into consideration and may provide a clearer result.

3.2.3. CEC mass balance in studied WwTWs

The estimated total mass of 119 of the 138 CECs in this work entering (quantifiable in total influent) the WwTW of this catchment is 1,185 kg per week (wk^{-1}) (or 1,847 kg wk^{-1} with creatinine). $Influent_{SPM}$ contributes only 0.8 % (9.6 kg wk^{-1}) of the total load, but as seen in Figures 1 and 2, it has a very different chemical profile. This results in total mass loads of 135 to 167 g d^{-1} 1,000 inh^{-1} in influent, these are far higher than the 2.1 g d^{-1} 1,000 inh^{-1} mass loads calculated from the work by Castiglioni et al., in Italy (based on the sum of the $influent_{AQ}$ loads of five main classes, 5,049 g d^{-1}, divided by the estimated population (2,400,000) of the contributing WwTWs)(Castiglioni et al., 2018). Though both studies cover a large range of pharmaceuticals, industrial chemicals and personal care products ingredients, Castiglioni's study only has 82 CECs, compared to 138 in this study, though both contain many similar high usage CECs. Furthermore, there are likely to be large differences in prescriptions and industrial contribution between Italy and the UK.

1,082 kg (1,696 kg, including creatinine) is removed from the $influent_{AQ}$ over the course of the study, leaving 72 kg (73 kg including creatinine) in effluent and entering the environment. 51 kg of this is from WwTW E which discharges directly into the estuary, which could not be sampled as part of this study. For the remaining WwTWs the highest contributor, by mass, was WwTW C with 11.6 kg discharged and leads to clear increases in daily river loads both downstream at WwTW C and upstream at WwTW D. The mass discharged by each WwTW generally increases by population equivalents contributing to the WwTWs i.e. WwTW B < WwTW C < WwTW E, however WwTW D, despite having around half the population of WwTW A, shows much higher mass discharge. Normalising the daily load discharge by each WwTW shows the highest population normalised loads are at WwTW D. WwTW A (5 g d^{-1} 1,000 inh^{-1}) < WwTW E (9 g d^{-1} 1,000 inh^{-1}) < WwTW B (12 g d^{-1} 1,000 inh^{-1}) < WwTW C (15 g d^{-1} 1,000 inh^{-1}) < WwTW D (16 g d^{-1} 1,000 inh^{-1} (21 g d^{-1} 1,000 inh^{-1} with creatinine)). Despite this, WwTW D removed the highest mass load per person, 151 g d^{-1} 1,000 inh^{-1}, which is close to WwTW E's removal at 146 g d^{-1} 1,000 inh^{-1}. Based on this TF and SBR show similar removal per person, however, as a proportion of the incoming load WwTW E removed 94.5 %, whereas WwTW D removed 90.4 %. Overall, WwTWs with TF appear to have a lower capacity for removal of CECs than

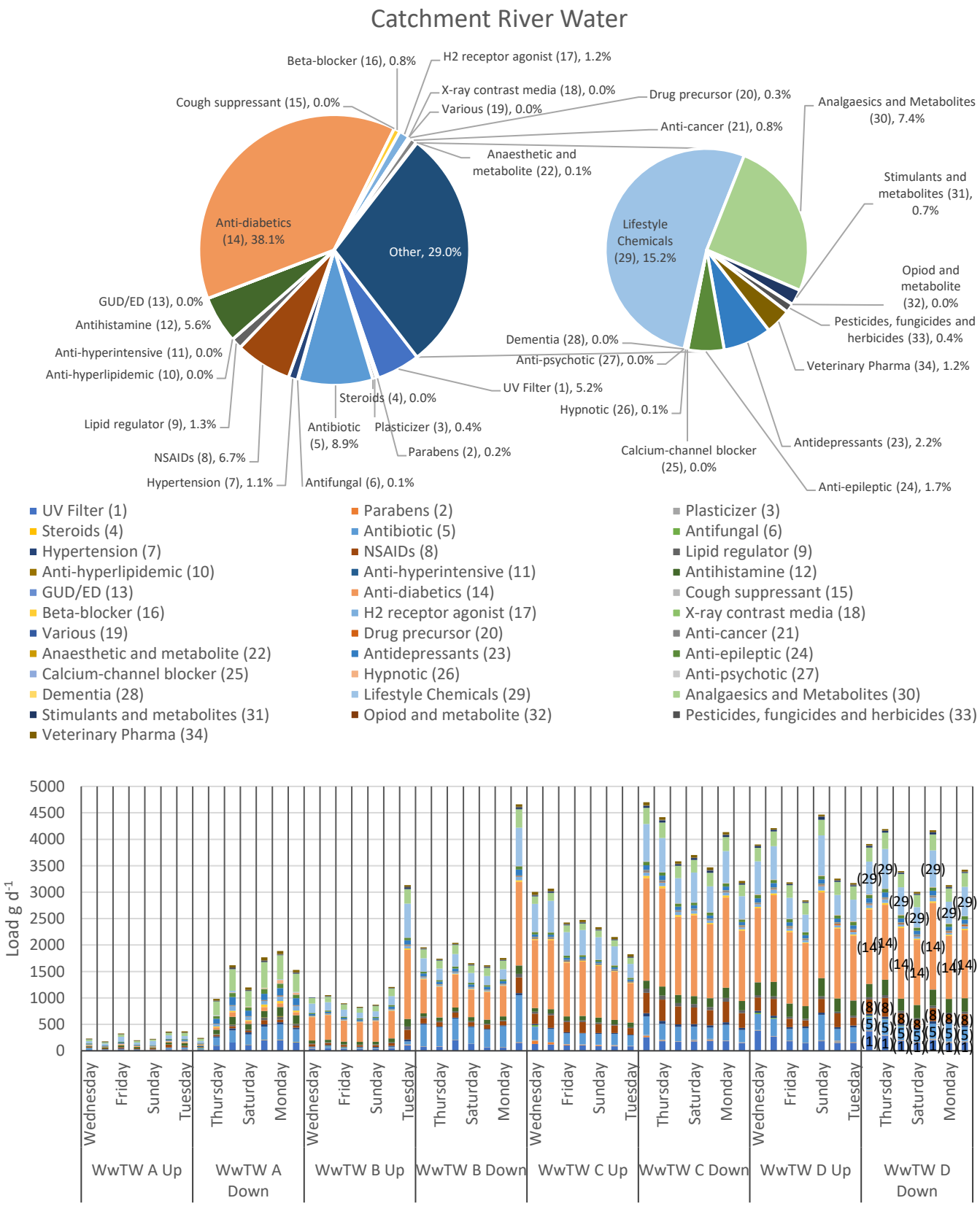


Figure 4 *Weekly percentage of total loads in river water of the entire catchment as a pie chart of classes, with chart showing spatial and temporal trends. Note: creatinine is not included. 0.0% shows negligible to no contribution to the total. Numbers in brackets indicate numbers assigned for identification in small figures and table*

SBR, (WwTWs B and C removed 78.1 % and 88.7 % respectively) whereas WwTW A appears to be the worst with 69.8 % total CEC mass removed. Although, WwTW A showed the lowest contribution with only 0.2 kg over the course of the study difference between upstream and downstream or 0.7 g d^{-1} 1,000 inh^{-1}. The small discharge into a large river at WwTW D, shows only a small difference between upstream and downstream of 0.4 kg over the course of the study or 3 g d^{-1} 1,000 inh^{-1} in the river. WwTW B and C had the highest increase in mass between upstream and downstream at 6.5 kg and 10.2 kg, or 14 and 13 g d^{-1} 1,000 inh^{-1}, respectively. Overall, the river upstream of WwTW A contained total mass loads of 1.8 kg, or 287 g d^{-1}, which increased to 25.2 kg, or 3.6 kg d^{-1} downstream of WwTW D (distance between A and D, is approximately 60 km). Throughout the catchment, 10.4 kg d^{-1} was discharged into the environment from the studied WwTWs.

3.3. Impact of effluent discharge to receiving river water

The river upstream of the WwTW A had 50/138 CECs above MQL ranging from 0.02 g d^{-1} (cocaine) to 47.8 g d^{-1} (caffeine), which is due to other smaller WwTWs present upstream, leaching from landfills sites, and possible infiltration from septic tanks, which are often used in more rural areas in the UK. Other classes such as plasticisers, veterinary pharmaceuticals, pesticides, fungicides and herbicides may possibly be present as well, due to surface runoff. Samples from the river downstream of the sites show higher loads overall, but also a different distribution of classes, with anti-diabetics, namely metformin, present at a larger proportion (from first being undetectable upstream of WwTW A, to representing 1,309.6 ± 135.5 g d^{-1} downstream of WwTW D). Daily loads ranged from 0.005 g d^{-1} (ketamine, WwTW A) to 1,890.3 g d^{-1} (metformin, WwTW C, equivalent to ~1,890 tablets (DrugBank, 2015)) for the 84/138 CECs that were detected downstream of the WwTWs. This trend of increasing load down the river is both expected, although perhaps not to this degree, and concerning.

Figure 4 (and Figure S5-6) show spatial trends of daily cumulative load and shows a steady increase down the river. Similar trends have been seen in Italy with samples which were collected in the River Lambro basin either side of Milan (Castiglioni et al., 2018). WwTW C is clearly the highest contributor to river load, which is not surprising as it has the highest population out of WwTWs A-D. The key classes of importance in river water are anti-diabetics, human indicators, NSAIDs, antihistamines, antibiotics, UV filters and analgesics and metabolites which contribute large portions to the total load with the river. This is interesting to compare with the distribution of classes within effluent, as analgesics and metabolites appear to contribute far more highly to effluent (21.0 %), however downstream from the discharge point they contribute far less, only 7.3 %. This indicates that once in the environment, they are far less persistent in the aqueous phase than other classes. A similar trend can also be seen for anti-depressants. Whether these compounds are truly degraded or have partitioned to solid phases (e.g. soils and sediments) within this river will need further investigation. However, a

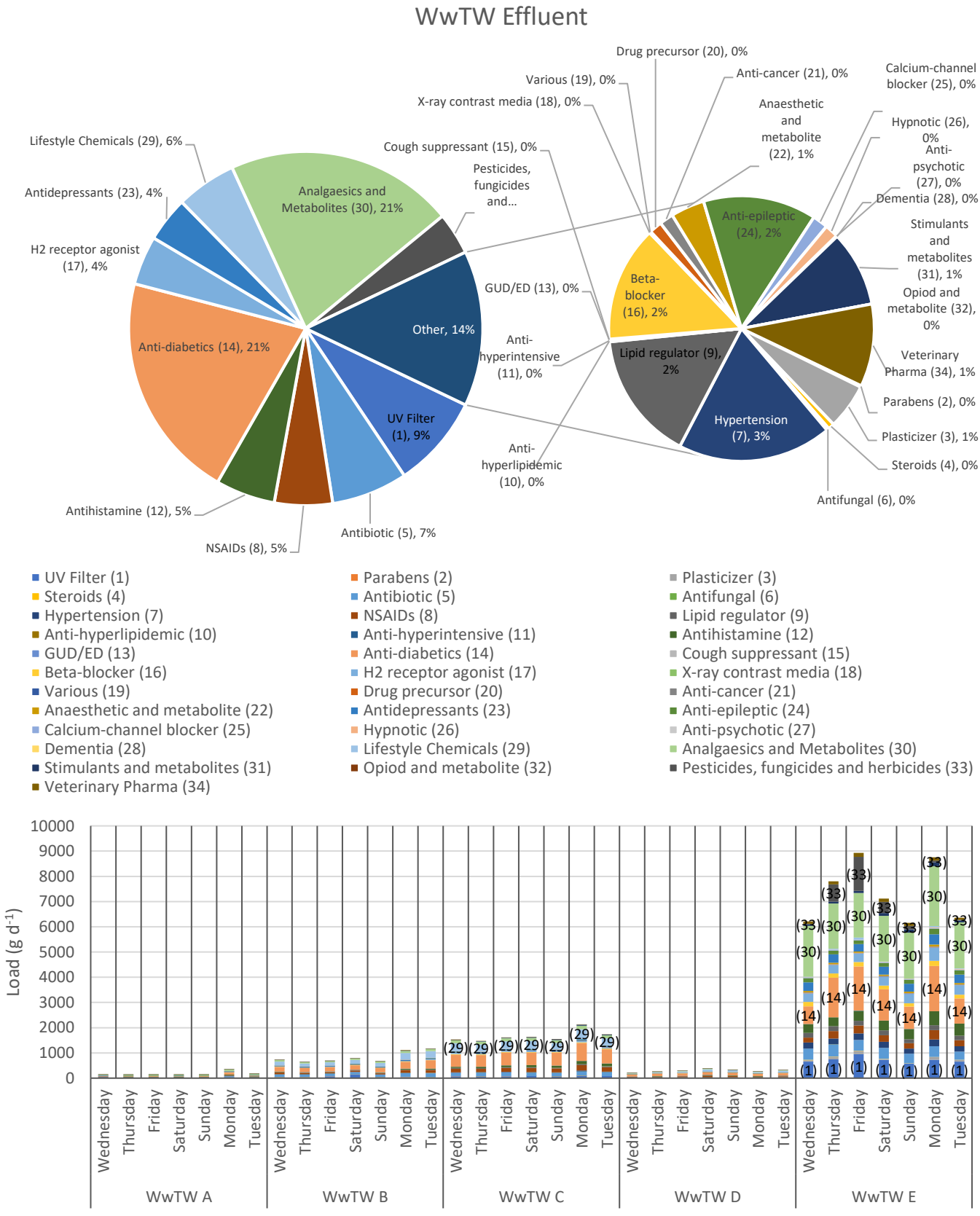


Figure 5 *Weekly percentage of total loads in effluent of the entire catchment as a pie chart of classes, with chart showing spatial and temporal trends. Note: creatinine is not included. 0.0% shows negligible to no contribution to the total. Numbers in brackets indicate numbers assigned for identification in small figures and table*

spatio-temporal study in the Llobregat showed that psychiatric drugs, among many other pharmaceuticals, were at levels ranging from 4.41 - 18.02 ng g^{-1} in sediment between the two sampling campaigns and locations. This may be indicative of portioning to solid phases within the river of this catchment. Sertraline in particular showed high concentration levels in Llobregat with 12.08 ng g^{-1} in one sampling campaign (Osorio et al., 2016). Furthermore, antibiotics, such as tetracyclines, will pose further concern as they have been shown to preferentially partition to sediment over surface waters (Kim and Carlson, 2007).

Anti-diabetics, metformin specifically, despite high level of removal (78.7 %), still represent a large proportion of effluent load (15.0 kg of 72.6 kg of the estimated total of the campaign, 20.7 % (Figure 5)). It shows that this removal level is insufficient in preventing anti-diabetics from entering the environment, as an increasing trend is observed through the catchment, as seen in Figure 6. A similar situation is seen for the lifestyle chemicals, which represents 38.6 % of the $influent_{AQ}$ load and despite their high removal rates they are at quantifiable levels in the environment and show an increasing trend through the catchment (Figure 6). This is less so for NSAIDs, which are similarly prevalent in $influent_{AQ}$, at 36.8 % of $influent_{AQ}$ load on average, but show less of an increase through the catchment. Diclofenac shows clear decreases in loads between sites, whether this is degradation or partitioning to solids, is yet to be determined. However, it has been previously found to partition to river sediments downstream of discharge points, along with other NSAIDs, therefore this fate seems likely within this catchment (Duan et al., 2013).

Benzophenone-3, methylparaben and propylparaben are shown to increase between downstream at WwTW B and upstream at WwTW C. For many other CECs, there is a slight increase suggesting the presence of another source of these compounds in the catchment. The increase of these compounds, associated with personal care products, could be due to much smaller WwTWs contributing to tributaries in the area, however, a similar increase in other CECs would also be expected e.g. carbamazepine, which is not seen. These CECs are usually found in greywater, i.e. from showers and washing. It is currently allowed, although not advised, for greywater from boats to be disposed of directly into the river. It is a practice that may be common in areas outside of marinas where disposal points are few and storage of wastewater onboard is limited and reserved for sewage (Canal and River Trust, 2017). Therefore, the presence of a large number of moorings in this area may contribute to this increase in personal care product ingredients. However, further investigation is required as both locations were not sampled as the same time and the use of grab sampling adds a level of uncertainty.

The river trends of flufenacet and oxadiazon show some small contributions from WwTWs, however the increase between downstream at one site and upstream at the next (particularly between WwTW B and C) supports entry is not primarily via WwTWs but further investigation would be needed to

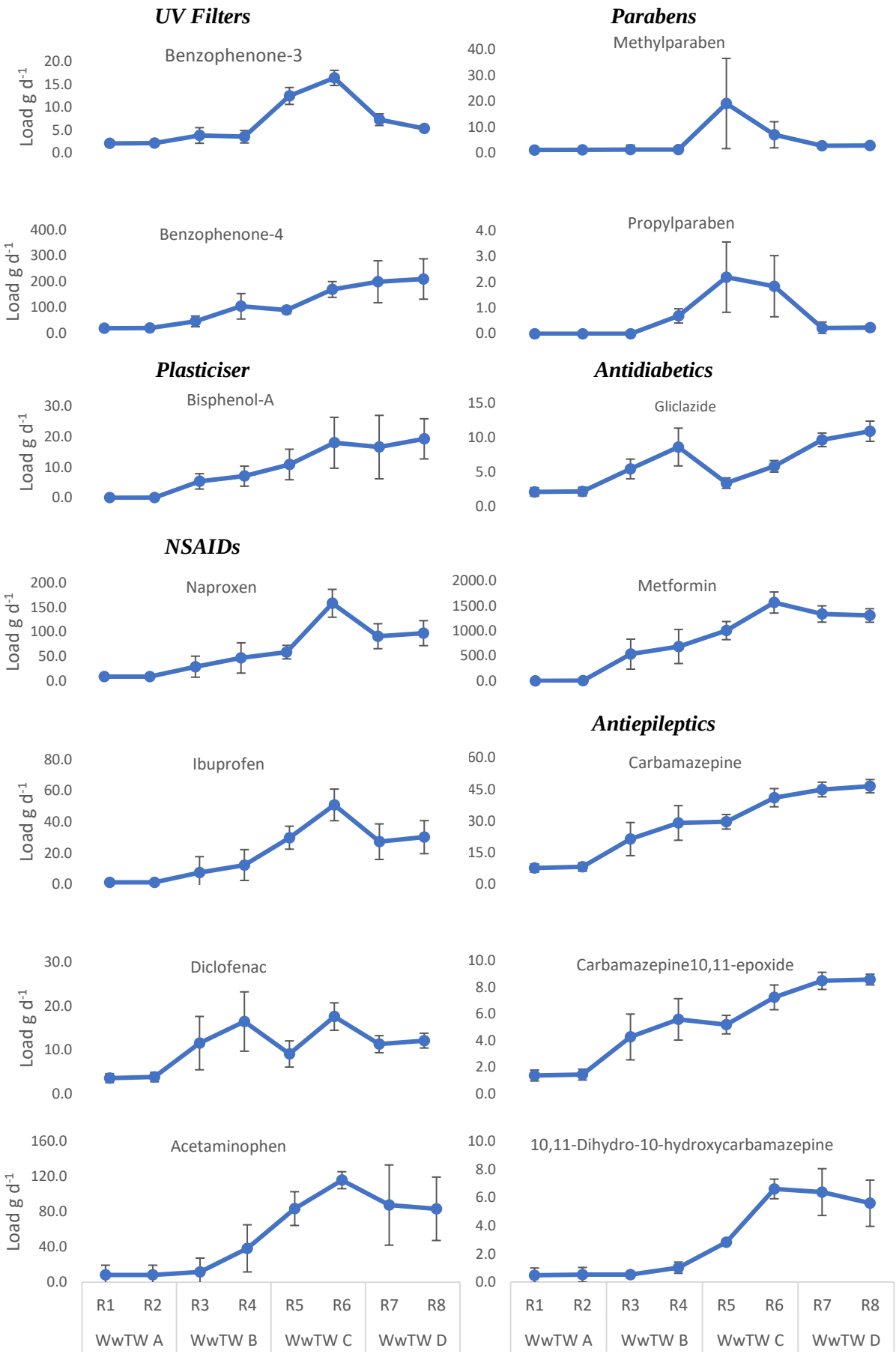


Figure 6 *Spatial trends down river for selected compounds. Note: Error bars indicate weekly variation of the sampling site*

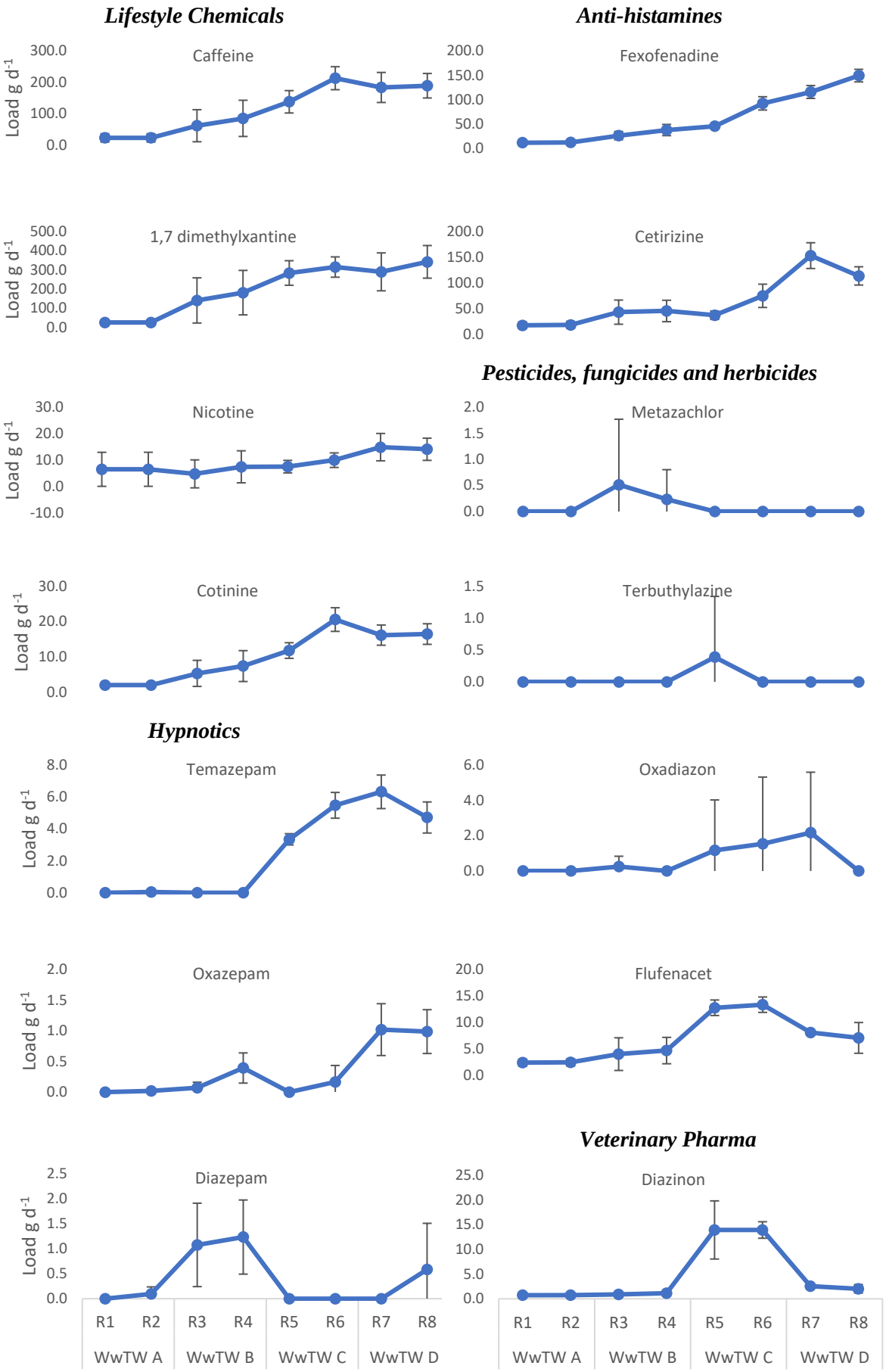


Figure 6 cont. *Spatial trends down river for selected compounds. Note: Error bars indicate weekly variation of the sampling site*

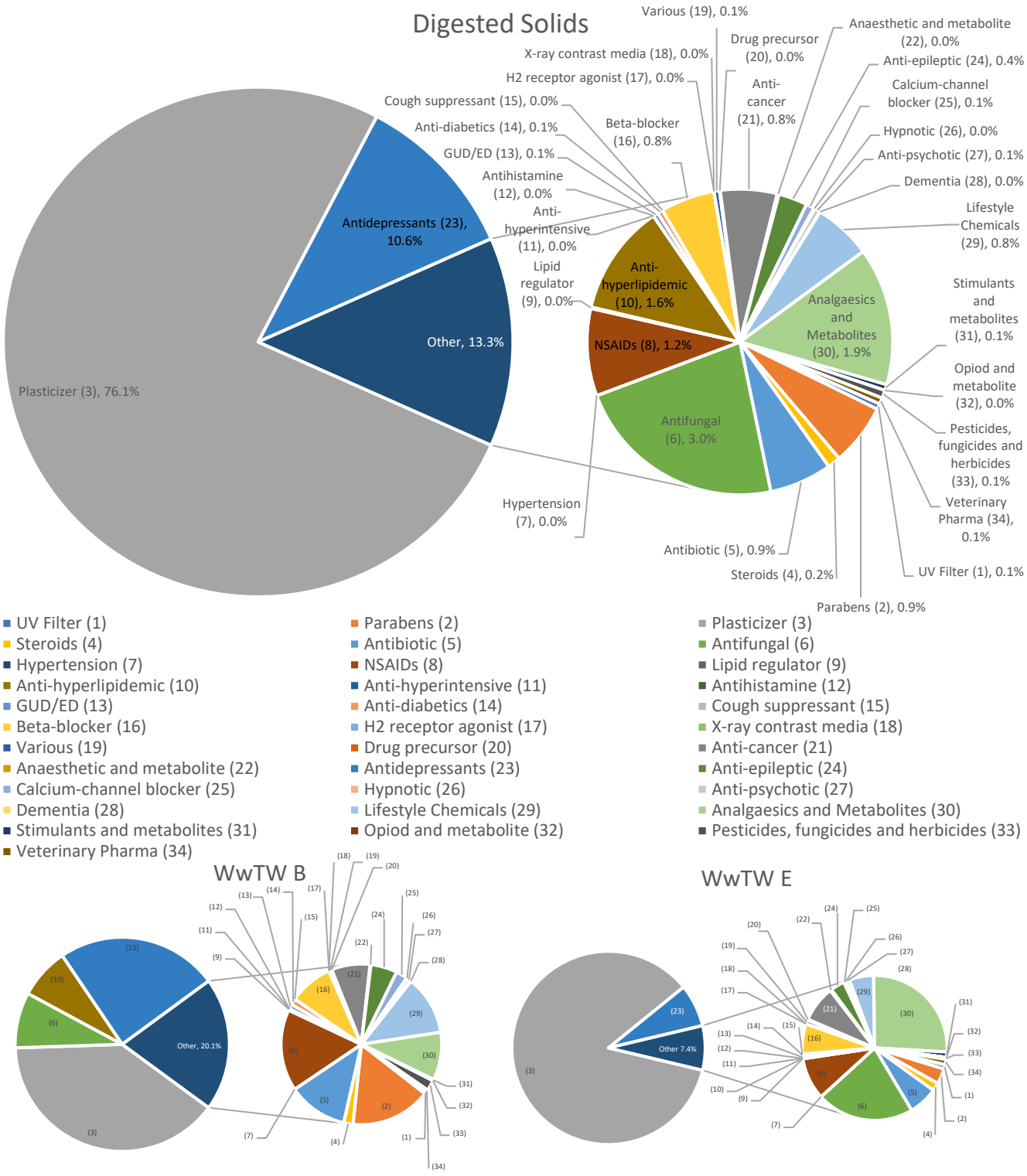
determine the source. Entry of pesticides into environmental surface waters has previously been attributed to diffuse sources such as agricultural application, particularly in proximity to surface waters and further surface runoff during wet weather (Lefrancq et al., 2017; Stuart et al., 2012). Due to the planning of the sampling campaign, rainfall and surface water runoff were at a minimum though this still seems likely to be a source, especially considering the level of agriculture and proximity of farming fields adjacent to the river throughout most of the catchment.

3.4. Presence of micropollutants in digested sludge for land application

An alternative route of entry for anthropogenic micropollutants into the environment is the application of digested sludge (biosolids) onto agricultural land. This area is often overlooked due to the additional analytical requirements to extract micropollutants from solid matrices and the lack of good analytical approaches available (Petrie et al., 2014b). Within the catchment, two WwTW sites had facilities for anaerobic digestion of sludge. WwTWs B and E both receive tankered and piped sludge (primary and secondary) from WwTWs within the catchment in addition to the sludge produced on site.

Digested sludge collected from WwTWs B and E was found to contain 65/96 different CECs (Table S5). This included NSAIDs (1.8 % of the total CEC concentration in digested solids (Figure 7)), antidepressants (10.6 %) and analgesics (1.9 %) which were ubiquitous in all samples studied. Ibuprofen, naproxen and diclofenac were all found in digested sludge, with ibuprofen at the highest concentrations for the class with 200 ± 42 ng g^{-1} dry weight (dw) at WwTW B. Although these concentrations are comparable to those previously reported (Guerra et al., 2014; Martín et al., 2012; Radjenović et al., 2009; Sabourin et al., 2012). Of the 12 antidepressants and metabolites studied and quantifiable in sludge, all were detected, including paroxetine and duloxetine which were found in no other samples throughout the catchment. This is attributed to their tendency to sorb to organic matter in wastewater and during treatment, as well as their recalcitrance in biologically mediated processes. Amitriptyline, sertraline and citalopram were present at concentrations > 400 ng g^{-1}. Morphine was the analgesic found at the highest levels with a mean concentration of 413 ± 43 ng g^{-1} at WwTW E. For such compounds, there is limited published data on their occurrence.

Other CECs found at notable concentrations (>100 ng g^{-1}) were methylparaben, BPA, chloramphenicol, ketoconazole, gemfibrozil, propranolol, carbamazepine and nicotine. Of these micropollutants, BPA was found at the highest levels with mean concentrations of 4,366 ± 260 ng g^{-1} (WwTW B) and 37,025 ± 4,229 ng g^{-1} (WwTW E) (Table S5). These concentrations are greater than has been observed in previous studies, which have found BPA at concentrations of ~1,000 ng g^{-1} (Langdon et al., 2014; Samaras et al., 2013) to 14,400 ng g^{-1} (carbon normalised concentrations) (Kinney et al., 2006). The levels reported here are attributed to the relatively high concentrations observed in receiving wastewater



% of total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
WwTW B	0.1	3.3	39.4	0.4	2.4	8.4	0.0	3.3	0.1	7.7	0.0	0.0	0.2	0.2	0.0	1.8	0.0	0.0	0.1	0.0	1.6	0.0	24.4	1.1	0.5	0.0	0.2	0.0	2.4	1.9	0.1	0.0	0.4	0.1
WwTW E	0.1	0.3	85.4	0.1	0.5	1.6	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.1	0.0	0.6	0.0	7.1	0.2	0.0	0.0	0.1	0.0	0.4	1.9	0.1	0.0	0.0	0.1

Figure 7 *Percentage of total concentration in digested solids of the entire catchment as a pie chart of classes, with individual pie charts for each site. Note: creatinine is not included. 0.0% shows negligible to no contribution to the total. Numbers in brackets indicate numbers assigned for identification in small figures and table*

from industrial activities. In this study, BPA contributed 76.1 % to the total concentration in digested solids.

As described by Carballa et al., and Hyland et al., several factors including physicochemical properties of both digested solid and the CECs, as well as the pH, temperature and water content may influence sorption of CECs to the digested solids (Carballa et al., 2008; Hyland et al., 2012). Crucially, the CECs present in digested solids, which have affinity with the aqueous phase, e.g. ibuprofen and naproxen, may not stay partitioned to the solids upon application of digested solids to the environment. These may enter landfill leachates or surface runoff from agricultural applications and may enter the aqueous environment via this route. Other CECs such as BPA show some recalcitrance in amended soils, possibly due to strong sorption and lack of bioavailability, leading to a lack of degradation as found in a fraction of BPA by Zhang et al., (Zhang et al., 2015).

4. Conclusions

This paper aimed to investigate the changes in micropollutants load throughout a river catchment system in the South-West of the UK, to gain further information on their sources, fate and behaviour. This was achieved by undertaking a comprehensive investigation of an extended list of 142 CECs at five strategic WwTWs representing >75 % of the wastewater from the catchment population. The main conclusions are as follows:

1. Lifestyle, availability of pharmaceuticals without prescription and industry have the biggest effects on the content of influent. Population size and the extent of urbanisation are key drivers of high variability across the catchment, and increased levels of CECs in the environment down the catchment. This is confirmed by normalisation of CEC loads for population results, which results in a more even distribution (154 ± 12 mg d^{-1} inh^{-1}).
2. The analysis of both aqueous and solid influent phases is key to determine true levels of CECs entering the works. Furthermore, each phase has a distinct chemical composition and some CECs may be found primarily in one phase or the other. Without analysis of both, a holistic understanding of pollutant fluxes is not possible.
3. Investigating temporal trends can highlight potential instances of incorrect use, incidental release or direct disposal. Although this is evident in both phases, it is particularly clear in the solid phase in this study, e.g. carbamazepine and ketoconazole. Furthermore, the current impact of these sudden, acute, events is currently unknown but may have noticeable effects on wastewater treatment processes or pose an environmental risk.
4. Despite WwTWs not being designed for the removal of CECs, the majority of the studied CECs were removed from the works to the high extent (10.3 kg d^{-1} remaining in effluent compared to

167.9 kg d^{-1} in influent). This markedly decreased the potential environmental burden posed by the extent of urbanisation and size of the population within this catchment.

5. Analysis of the river water upstream and downstream of the WwTW discharge point allowed the contribution of each WwTW to the environmental burden to be considered. It also highlights the potential for contribution to the environmental burden from other sources, which may include: septic tanks, sewer overflows, smaller WwTWs, surface runoff and greywater disposal. Furthermore, it showed that many CECs are ubiquitous throughout the catchment, with many increasing in load down the river due to the persistent addition of these compounds to the environment being higher than their degradation rate.
6. Analysis of digested solids has shown high levels of a wide range of CECs present (65/96). These concentrations are significant and considering the potential use of this 'treated' matrix in amended agricultural soils, further consideration should be given to the potential ecological risk of this matrix, which is currently barely understood. Furthermore, the removal trends/treatment efficiency require further study.

Acknowledgements

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Supplementary material:

Section S1 describes the material and methods used in this work in more detail.

Table S1 shows the instrumental and method performance data for the analytical method.

Section S2 describes the data processing that was used in this work

Section S3 add some additional information to the results in the main text for additional context or more indepth discussion

Table S2 shows the dilution factor of effluent in the river water at the discharge point at each site.

Table S3 provides the 7-day mean population normalised loads for $influent_{AQ}$.

Table S4 provides the 7-day mean population normalised loads for influent$_{SPM}$.

Table S5 presents the frequency of detection of each analyte, the minimum and maximum loads, the mean, standard deviation and variance across the 7 days in each matrix.

Table S6 shows the 7-day average percentage partitioning of CECs in influent for all sites.

Figure S1 presents the 7-day average percentage removal from influent$_{AQ}$ during WwTW treatment for each site and overall.

Table S7 shows the data of Figure S1, i.e. 7-day average percentage removal from influent$_{AQ}$ during WwTW treatment for each site and overall.

Table S8 shows the general and chemical information of all CECs analysed.

Figure S2 shows the temporal trends in influent for selected compounds.

Figure S3 shows the temporal trends in both influent$_{AQ}$ and influent$_{SPM}$ for selected compounds.

Figure S4 shows the spatial trends in river water through the catchment as cumulative load by class.

Figure S5 shows the spatial trends in river water through the catchment as cumulative load by individual CEC.

Table S9 shows the metadata of the sampling campaign, i.e. daily temperature, rainfall and pH of samples.

Section S4 shows the references for the SI.

Tables S10-32 shows the detailed daily loads (g d^{-1}) for each CEC at each site in each matrix.

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Appendix 6B: Statement of Authorship

This declaration concerns the article entitled:			
Catchment based approach to environmental risk assessment			
Publication status (tick one) **Draft manuscript** [X] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [ ]			
Publication details (reference)	Proctor K, Barden R, Kasprzyk-Hordern B, et al. Catchment based approach to environmental risk assessment. (In preparation).		
Copyright status (tick the appropriate statement) I hold the copyright for this material [X] Copyright is retained by the publisher, but I have been given permission to replicate the material here [ ]			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: The candidate was the major contributor in how the environmental risk assessment approach was applied to the catchment-based study Design of methodology: The candidate was the major contributor in the research and design of the catchment-based environmental risk assessment of both aquatic and terrestrial environments Experimental work: The candidate was one of the major contributors in the original sample preparation of solid and liquids samples and their analysis. The candidate was the main contributor in the collection of ecotoxicity data, application and evaluation of the environmental risk assessments. Presentation of data in journal format: The candidate was the major contributor for the data analysis, interpretation, and presentation of the data in this paper.		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	Kathryn Proctor	**Date**	11/12/2020

Chapter 4

Catchment based approach to environmental risk assessment

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Abstract

Chemicals of emerging concern (CECs) have been found throughout the environment and around the globe. Efforts need to be taken in understanding the risk they pose to the environment. The environmental risk of individual CECs is of little use when considering the complex mixture of CECs present in the environment. This paper considers the acute and chronic environmental risk to aquatic and terrestrial organisms based on measured environmental concentrations from a comprehensive catchment study.

For the aquatic environment, the mixtures present a high acute risk, which varied across the catchment, with an overall trend of increasing risk down river, with a risk quotient ($RQ_{MEC:PNEC}$) of 32.1, at the furthest sampled point upriver, to 106.0 at the furthest point downriver. There was elevated chronic aquatic risk with $RQ_{MEC:PNEC}$ of 99.4 upriver to 516 downriver at the same sampling locations. The 'sum of toxic units' approach showed the algal trophic level was most at risk from acute toxic effects, whereas the daphnid trophic level was most at risk from chronic toxic effects. This work found most of the risk, >90 %, was contributed by a small number of compounds; bisphenol A and diazinon.

For the terrestrial environment, there was not enough publicly available ecotoxicity data for a full mixture based environmental risk assessment. Consideration of the data that was available, for the 21 out of 65 quantified CECs, shows there is little risk from amended soils to earthworms based on the predicted environmental concentration of soil. However, the digested solids themselves show a high acute and chronic risk for several CECs. Therefore, there is potential for localised pockets of high toxicity if the soils are not well mixed.

1. Introduction

Micropollutants have been shown to be prevalent, usually in the range of ng L^{-1} to µg L^{-1}, in many different environmental compartments, including surface waters, marine water, sediment and soils, as well as entering the food chain via lower trophic levels and bioaccumulation to higher levels. This is a global problem [1, 2] and efforts are being made by the European Commission to prioritise which of the 1000's of compounds (REACH, EINECs) are of highest risk to the aquatic environment and which pose an unknown or high level of risk and require more data [3–6]. Despite the focus on aquatic environments, other environments may also be at risk.

The risk micropollutants pose to the environment is complex, as rarely are the concentrations of individual micropollutants high enough to cause acute toxic effects. The problem lies within their potential to cause long-term chronic effects, both individually and as a mixture. For this reason, they have begun to be referred to as chemicals of emerging concern (CECs) [1, 7, 8].

It is widely understood that the ecotoxicity of individual compounds is often less than the toxicity of the overall mixture [9] and better understanding can come from reviewing the components in a mixture [10–12]. Much of the current environmental risk assessment (ERA) work has been focused on small numbers of compounds or a single class, e.g. antihypertensives [7], quinolone antibiotics [10], pharmaceuticals [13, 14], UV filters [15], pesticides [16] etc. Although a recent study was published looked at 47 CECs [17].

Currently ERAs typically follow the European Medical Agency guidelines for individual pharmaceuticals i.e. calculating predicted-no-effect-concentrations (PNEC) from effect concentration levels (EC50, EC10 etc) and relevant assessment factor (AF), and comparing with the predicted environmental concentration (PEC), for the relevant environmental compartment[18]. This method maybe useful for understanding the potential effects of a single compound, however the environment is far more complex than that and analysis of the environmental risk of mixtures is critical. However, the work by Backhaus and Karlsson, found the overall toxic effect of the 26 pharmaceuticals was primarily the contribution of a one or two compounds in most cases. This has been found with pesticides on several occasions by Verro et al. [19], who found no more than three pesticides caused >80% of the toxic effect, though this was based on PECs, and Gustavsson et al, [16]. found a similar situation over 11 years across six sites. However, they found that monitoring of 44 pesticides was still essential to account for at least 95% of the risk over this time period. A further study was carried out on Swedish coastal waters, by Gustavsson et al. [20], found 62 of the 172 compounds analysed were detected, at 3 of the 5 sites the majority of the risk to the environment was posed by triclosan, at the other two the majority of the risk was more evenly distributed between three or four compounds. Overall, this shows

there is a need to analyse a wide variety of CECs to fully determine the risk to the environment, as a small number of compounds may be responsible for the majority of the risk, but they may not be identified if the scope of analysis is too narrow. In these studies, the compounds responsible for the majority of the risk have been shown to fluctuate with spatial and temporal variation.

This work aims to provide the most comprehensive catchment based ERA to date, using measured environmental concentration (MEC) data from seven consecutive days at each of the 8 sites along a river considering spatial and temporal variations. Furthermore, a theoretical risk assessment of the application of digested solids, from two WwTWs within the catchment, to agricultural soils for terrestrial organisms is also carried out. This work considers the risk of CECs individually, as a mixture by class and as a total mixture to both the aquatic and terrestrial environment.

2. Materials and Methods

2.1. Materials

All materials used in the investigation are detailed in the Supplementary Information (Section S1) or in the paper by Proctor et al., [21] (Chapter 3).

2.2. Targeted chemical of emerging concern (CECs)

The 142 CECs cover 30 classes in this paper, including, industrial chemicals, personal care products, pharmaceuticals for veterinary and human therapy, pesticides, herbicides and fungicides. They were selected based upon a review of current and proposed EU and national regulation and legislation and literature [3, 5, 6, 22, 23], as well as information on usage i.e. prescription data for pharmaceuticals [24], expert knowledge on pesticide use and data from literature for veterinary pharma [25–27], metabolism data (Drugbank [28], toxicity to mammals, aquatic and benthic organisms and potential persistence, bioaccumulation, transport through the environment based on physiochemical parameters such as Log K_{ow}, Log $D_{ow,}$ water solubility, and bioconcentration factor (BCF), from EPI Suite and ACD/Labs [29, 30]. They were prioritised for their potential to reach the environment and cause a negative impact. The general chemical information and physicochemical parameters can be found in the Supplementary Information, Table S1.

2.3. Studied catchment and sampling locations

The area studied in this work is a river catchment in the South West of the UK, covering approximately 2,000 km^2. The river is approximately 120 km in length and has an average discharge of 20 $m^3 s^{-1}$ and accepts discharges from several WwTWs covering a population of ~1.5

million. The catchment also contains private treatment facilities, such as septic tanks. River sampling points were chosen upstream and between 0.5 to 2 km downstream of the discharge points 4 of these WwTWs. These are labelled R1 to R8 throughout, R1, R3, R5, and R7 were upstream of WwTW A, B, C and D respectively and R2, R4, R6, R8 were downstream of the WwTWs respectively as shown in Figure 1(further site information can be found in Supplementary Information, Table S2). At the most upstream WwTW, A, sampling downstream was shown to have poor mixing in the river and higher than expected environmental concentrations). For this site, R2, PECs were calculated based upon river upstream concentrations, effluent concentrations and dilution as discussed in section 2.7. Dilution of the effluent in the river was between 12.6 ± 1.4 for WwTW B and 153.3 ± 23.9 for WwTW C. Further effluent dilution information can be found in Supplementary Information, Table S3. As previously calculated, in a previous study these four WwTWs were found to contribute 3.0 kg d^{-1} to the river, with between 0.18 kg d^{-1} (WwTW A) to 1.7 kg d^{-1} (WwTW C). River samples for WwTW E, the final and largest WwTW, treating wastewater equivalent of ~850,000 people, could not be collected as the effluent is discharged to the estuary and samples could not be collected safely. Flow data for effluent discharge and river water can be found in the Supplementary Information, Table S4.

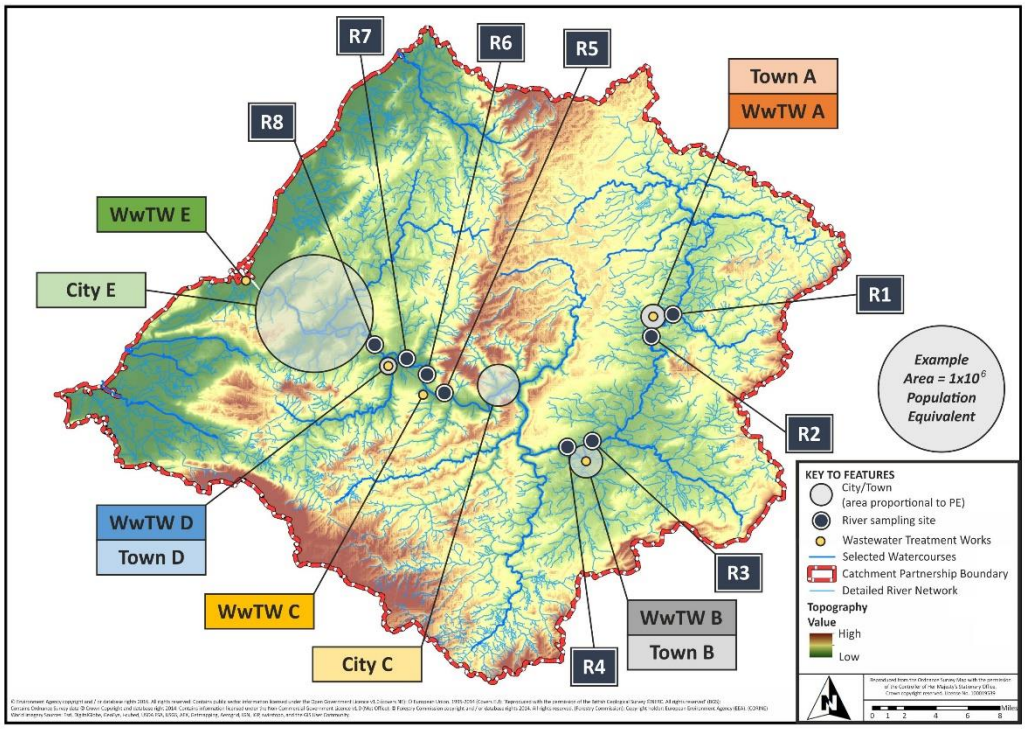


Figure 1 Catchment map showing, city/town, WwTW and river sampling site locations. The circled area around each town/city represents the population size.

2.4. Sample collection

Samples of the liquid and solid phases of influent, effluent and river water upstream and downstream were collected on seven consecutive days at each WwTW. 24-hour volume proportional composites were collected for influent and 24-hour time proportional composite samples were collected for effluent. The composite samplers collected subsamples of 80 mL approximately every 15 minutes. The samplers were stored on ice within the sampler to maintain a low temperature and prevent degradation of the analytes. Surface water was sampled via grab sampling each day to provide accurate concentrations, without needing to consider degradation or dilution within composites. All samples were placed on ice whilst being transported to the lab.

2.5. Sample preparation

Samples were prepared following the method discussed by Proctor et al., [21, 31]. To summarise, all samples were filtered with GF/F glass fibre filter paper, before measuring 100 mL and spiked with 50 µL of 1 µg mL^{-1} of internal standard (IS) mix. The samples were then loaded onto equilibrated HLB (hydrophilic lipophilic balanced) solid-phase extraction cartridges, before being eluted with methanol (MeOH) and evaporated to dryness with a Turbo evaporator under nitrogen. The sample was then reconstituted with 500 µL of 80:20 H_2O:MeOH, mixed thoroughly and transferred to LC (liquid-chromatography) vials for analysis.

Once at the lab digested solids were frozen at -20˚C, before being freeze dried and homogenised. The resultant powder was carefully weighed to 0.25 g and spiked with 50 µL of 1.0 µg mL^{-1} internal standard mix. This was then combined with 25 mL of 50:50 deionised water:acidified MeOH (methanol) (pH 2) in a microwave assisted extraction (MAE) tube and shaken well. The tubes were placed in the 800 W MARS 6 microwave and held at 110 ˚C for 30 mins. Once cool, the samples were filtered and the filtrate was loaded onto conditioned and equilibrated MCX (mixed-cation exchange) solid-phase extraction (SPE) cartridges. The analytes were eluted in two fractions. The acidic analytes were eluted first with 2 mL of 0.6% formic acid in MeOH, then the basic fractions were eluted with 3 mL of 7% ammonium hydroxide in MeOH. Both fractions were then evaporated to dryness at 40 ˚C under nitrogen, before being reconstituted in 500 µL 80:20 deionised water:MeOH and mixed thoroughly. These were then transferred to LC vials for analysis.

Analysis was carried out as described by Proctor et al. [31] (Chapter 2), specifically using a Waters ACQUITY UPLC™ (ultra-performance liquid chromatography) system coupled to Xevo Triple Quadrupole Mass spectrometer. Separation of analyte was carried out with a reversed phase C18

column (150 mm x 1.0 mm, with a particle size of 1.7 µm) and the mobile phase and gradient was dependent upon whether electrospray ionisation (ESI) was in positive of negative modes.

2.6. Analytical methodologies

All samples were analysed with ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). The parameters for the methods used can be found in the method validation paper by Proctor et al [31] (Chapter 2). Method detection and quantification limits (MDLs and MQLs), accuracy and precision can be found in Supplementary Information, Table S5.

2.7. Quality Control

Spiked quality control samples were analysed for each matrix at each site, at concentrations of 100 and 1000 ng L^{-1}. Matrix suppression effects and losses of analyte during sample preparation were counteracted by the use of IS. Identity of analytes was confirmed with the use of two transitions, retention time (± 20 %), and ion ratio (± 20 %) [32].

Thorough mixing of the effluent discharge and river water downstream is required to ensure accurate environmental concentrations are detected. For this reason, mass balances were compared for carbamazepine load downstream, and the sum of upstream and effluent (Equation 1), as previously described by Proctor et al [21] (Chapter 3). With thorough mixing, the difference is assumed to be negligible. As mentioned in Section 2.3., site R2 showed poor mixing, therefore predicted environmental concentrations based on the sum of effluent/dilution and river water upstream were used instead. Results for this can be found in the Supplementary Information, Section 2.

$$Mass\ balance\ (\%) = \left(\frac{Downstream\ river\ load\ (g\ d^{-1})}{(Upstream\ river\ load\ (g\ d^{-1}) + effluent\ load (g\ d^{-1}))}\right) x100 \quad (1)$$

2.8. Environmental Risk Assessment

2.8.1. Aquatic Ecotoxicity data

Modelled acute and chronic ecotoxicity data was collected for three trophic levels (algae, daphnid and fish) from ECOSAR for all 138 CECs quantifiable in surface water. This was supplemented with publicly available, experimentally derived acute and chronic ecotoxicity data, i.e. EC10, or NOEC or LOEC if well defined, for 34 and 36 CECs, respectively, from

selected classes; lifestyle chemicals, analgesics, anti-diabetics, anti-epileptics, NSAIDs, UV filters and antidepressants. This data was acquired from Web of Science search, following a method previously implemented by Johnson et al., [33], more information can be found in Supplementary Information, Section 3. Further searches were carried out from the ECHA – REACH and TOXNET HSDB databases. In instances where the source of the data, i.e. the paper, could not be identified the database is given as the reference. The collection of papers was vetted for organism class, species, model/lab/wild organism source, test protocol, duration, well known endpoints, EC10, EC25, EC50, NOEC, LOEC units and references. Attention was made to NOEC and LOEC values and these were not used if the dose-response curve was not adequately characterised.

Both acute and chronic toxicity data was used for comparison as EC50 ecotoxicity data is more commonly used but chronic toxicity data is more relevant to the environment. Furthermore, this approach has recently been advised in the newly amended EMA environmental risk assessment [18].

2.8.2. Aquatic environmental data

As previously discussed, the aquatic environmental data was be obtained from the daily grab sampling that were collected over seven consecutive days at each site. Analysis of these samples will provide variation or consistency of the MECs over a week and across the catchment.

2.8.3. Soil Ecotoxicity data

Due to the lack of available experimental chronic toxicity data available for the majority of these compounds (publicly available experimental chronic toxicity data for only 9 out of 96 CECs quantifiable in digested solids were found), modelled chronic ecotoxicity data from ECOSAR were also used, however this could only be obtained for 23 CECs out of the remaining 87 (total of 32 compounds with data out of 96).

2.8.4. Soil Environmental data

Furthermore, the environmental concentrations within the soil (PEC_{SOIL}) are predicted based on the concentrations present in the digested sludge (C_{sludge}) and the equation (2) using default values from 'Guideline on the environment risk assessment of medicinal products' by the European Medical Agency [18]. Where $Appl_{sludge}$ is the yearly sludge application rate to soils (0.5 kg m^{-2}), Depth is the mixing depth (0.2 m), and Density is the bulk density of the wet soil (1,700 kg m^{-3}).

$$PEC_{SOIL} = \frac{C_{sludge\,(ng\,g^{-1})} \times Appl_{SLUDGE}\,(kg\,m^{-2})}{Depth\,(m) \times Density(kg\,m^{-3})} \quad (2)$$

2.9. Predicted No Effect Concentration (PNEC) calculation

PNECs were calculated using equation 3, as in the method described by Backhaus et al, EMA and European Commission TGD [18, 34, 35].

$$PNEC\ (ug\ L^{-1}) = \frac{lowest\ EC10\ (ug\ L^{-1})}{AF} \quad (3)$$

Where the '*lowest EC10*' is the lowest EC10 from the literature or modelled ecotoxicity data of the three trophic levels, and the AF is the assessment factor. This factor is applied to allow a more conservative environmental risk assessment, allowing more protection for the environment. Overall, with increasing confidence in the ecotoxicity data the AF should decrease. For acute ecotoxicity data i.e. LC50, an AF of 1000 is often used, due to the difference between acute toxic effects and the potential sublethal effects that may be seen at environmental concentrations. The advice based on Backhaus et al, EMA ERA and European Commission TGD, suggests an AF of 100 should be used for one chronic EC10 or NOEC for fish or daphnia, an AF of 50 for two data on two trophic levels, and an AF of 10 based on three trophic levels. For sensitive species distribution data, an AF of 5-1 should be used [18, 34, 35].

2.10. Risk Quotients

Risk quotients are a measure of environmental risk based on the ratio of PEC or MEC and PNEC. This paper uses the average MECs for each site, from a comprehensive dataset of MECs for 138 CECs, at 8 sampling locations in a river catchment for ERA of the aquatic phase and PEC_{SOIL} data calculated from 96 CECs in digested solids from two WwTWs within the catchment, providing a higher degree of confidence.

As suggested by Backhaus et al., there are two ways to calculate the RQ of the mixture considering concentration addition:

$$RQ_{mixture} = \sum_{i=1}^{n} \frac{MEC_i}{PNEC_i} = \sum_{i=1}^{n} \frac{MEC_i}{\min\left(EC50_{algae}, EC50_{daphnids}, EC50_{fish}\right)_i \times (1/AF_i)} \quad (4)$$

$$RQ_{STU} = \sum_{i=1}^{n} \max\left(STU_{algae}, STU_{daphnid}, STU_{fish}\right) \times AF$$

$$= max\left(\sum_{i=1}^{n}\frac{MEC_i}{EC50_{i,algae}}, \sum_{i=1}^{n}\frac{MEC_i}{EC50_{i,daphnids}}, \sum_{i=1}^{n}\frac{MEC_i}{EC50_{i,fish}}\right) \times AF \quad (5)$$

RQs have been interpreted in different ways from paper to paper, with Backhaus and EMA suggesting there is a risk or the environmental quality standard is exceeded if RQ >1 [18, 34]. Sousa et al., suggests that an RQ <0.1 is low risk, 0.1 – 1.0 is medium risk, >1 is high risk [1].

As this work uses MECs rather than PECs and considering the multitude of CECs present in mixtures within the environment, this work assumes RQ < 0.1 is low to no risk, 0.1- 1.0 is medium risk, and >1 is a risk.

Furthermore, this work calculates the RQ for several different aspects:

1. $RQ_{MEC:PNEC}$ - a measure of the risk of MEC to the environment
2. $RQ_{MQL:PNEC}$ – a measure of the risk of the lowest quantifiable concentration for compounds which were below MQL. Provides information on the potential risk on unquantifiable levels of these CECs.
3. $RQ_{mixture}$ – a measure of the risk of a mixture to the environment. This is discussed for classes throughout as well as the overall risk of the mixture as a whole
4. RQ_{STU} – another measure of the risk of a mixture to the environment as described by Backhaus et al, based on the Sum of Toxic units (STU) for each trophic level.

3. Results and Discussion

3.1. Occurrence in river water and aquatic environmental risk assessment

These results are based upon the average concentrations from a week sampling at 8 sites, (total n = 56 per compound). Figure 2 shows the distribution of classes in the average samples from each river sampling site. Figure 3 shows the average daily cumulative total for each river sampling site as a distribution of classes and as a distribution of CECs. More detailed information, on the average concentration of each CEC, at each site can be found in Supplementary Information, Table S6. Acute and chronic ecotoxicity data for three trophic levels, AF and PNEC discussed in the text below can be found in Tables 1 and 2. Acute and chronic $RQ_{MEC:PNEC}$ for the 8 sampled sites down the river are displayed as a heat maps and can be found in Tables 3 and 4. Followed by the acute and chronic $RQ_{mixture}$ for each class of CEC in Tables 5 and 6.

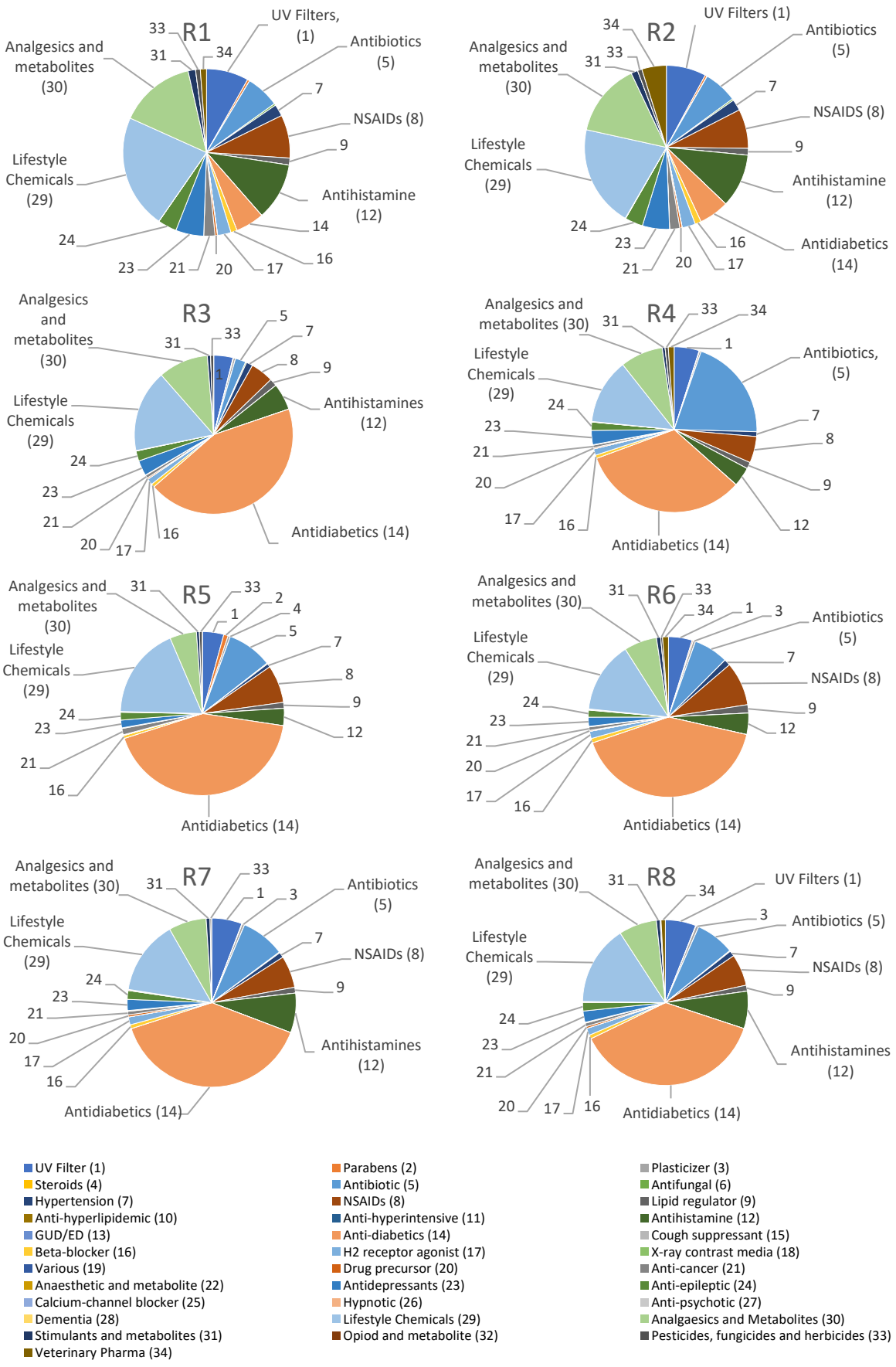


Figure 2 Pie charts for the distribution of classes in river water samples at each site, based on average over the week. (Number in brackets is for identification of class)

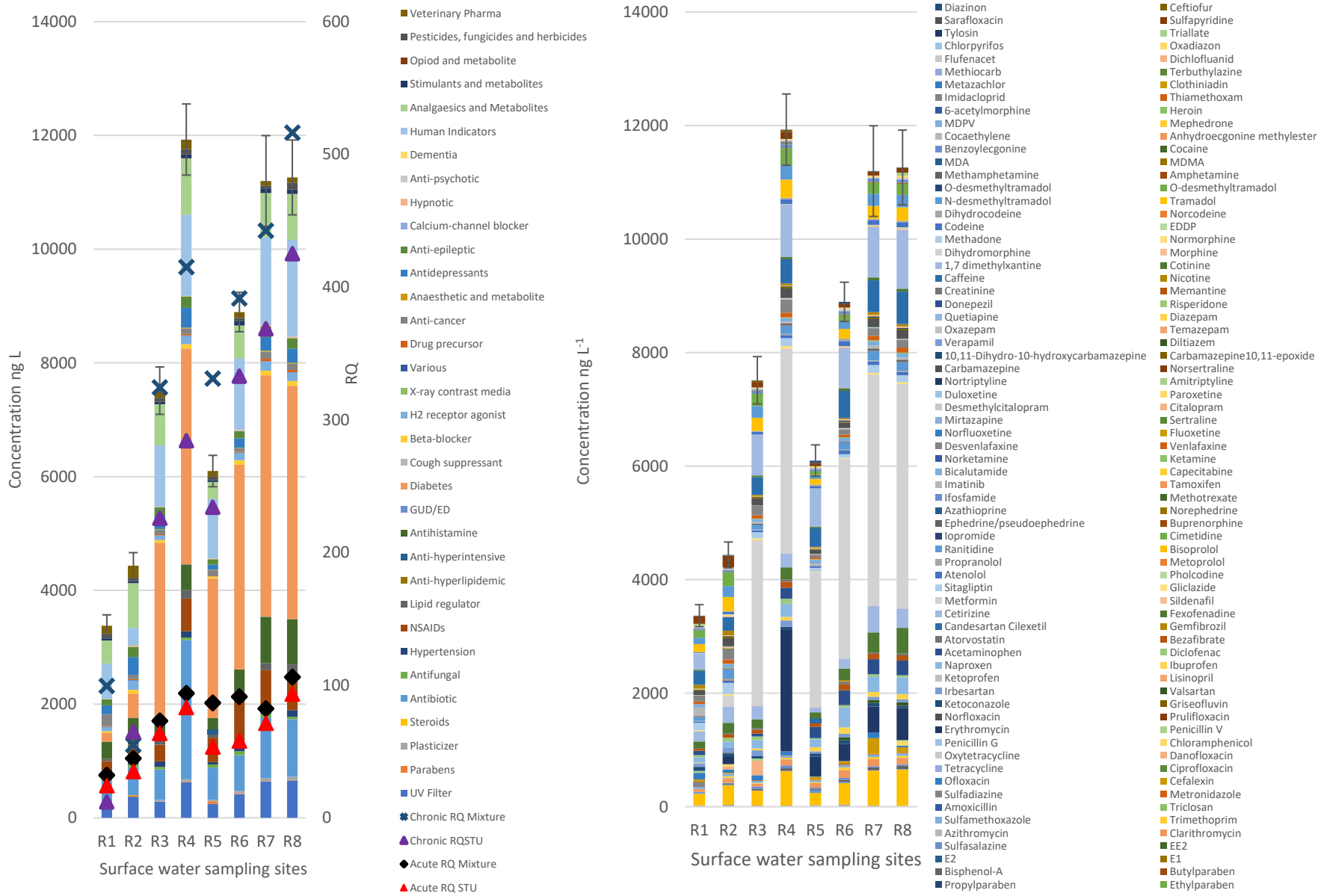


Figure 3 Average daily cumulative total for each river sampling site as a distribution of classes and as a distribution of CECs

The paper by Proctor et al., identified the UV filters, NSAIDs and acetaminophen, antidiabetics, antidepressants, anti-epileptics, lifestyle chemicals, and analgesics to be of particular interest for further investigation into potential environmental effects within this catchment due to their high levels and these CECs represent the majority of the CECs in river water [21].

3.1.1. UV filters

Benzophenone-1 and benzophenone-2 concentrations were too low to detect in river water throughout the catchment (MQL for benzophenone-1 = 0.35 ng L^{-1} and benzophenone-2 = 0.79 ng L^{-1}). Benzophenone-3 was found at concentrations ranging from 13.8 to 46.3 ng L^{-1}, whereas benzophenone-4 was found at concentrations ranging from 184.6 to 1,321.5 ng L^{-1}. The levels seen for benzophenone-3 are very similar to the predicted environmental concentration for the UK, 24 ng L^{-1}, by Dhanirama et al [36]. Benzophenone-3 was found at similar concentrations to those previously found in the UK, from <MQL (15 ng L^{-1}) to 44 ng L^{-1} , whereas the levels previously found for benzophenone-4 are much lower, from <MQL (3 ng L^{-1}) to 371 ng L^{-1}, though they were not detected as frequently [37].

With regards to their chronic ecotoxicity to aquatic organisms, benzophenone-2 is the most toxic with a PNEC of 0.4 µg L^{-1}, the others are ranked as such: benzophenone-3 (18.0 µg L^{-1}) > benzophenone-1 (32.7 µg L^{-1}) > benzophenone-4 (97.9 µg L^{-1}). Though the acute ecotoxicity data provides a lower PNEC, is it deemed to be less accurate due to the higher assessment factor applied.

Chronic risk quotients for benzophenone-4 shows the highest $RQ_{MEC:PNEC}$ with 0.007 (R8), which shows there is a very low risk to three trophic levels based on the standard approach. Considering the UV filters in this study as a mixture, the highest $RQ_{MEC:PNEC}$ across the catchment is 0.007 (R7). The sum of toxic units shows the daphnid/crustacean trophic levels is most at risk from this group of CECs. This is a lower risk than what was given by Riva et al [17], however, this is likely due to using chronic ecotoxicity data and a lower assessment factor . Both studies show limited risk to the aquatic environment from these compounds. However, this is in contradiction to Wu et al. [15], who found RQs of 2.7 and 0.8 for benzophenone-4 and benzophenone-3 at similar concentrations (up to 131 ng L^{-1} and 30.0 ng L^{-1}, respectively), likely due to an overestimation of hazard.

Whilst this appears not to have any chronic freshwater effects, the potential bioaccumulation of benzophenone-2 has been shown in freshwater fish by Díaz-Cruz et al [38] and

benzophenone-3 has been highlighted in two marine species, cod and prawn, by Langford et al [39], suggesting that testing the BAF in aquatic organisms is a key parameter for this class. This is especially important, considering the potential for hormonal and reproductive effects in fish [40]. Currently these CECs are not included in any monitoring programs [41].

3.1.2. Parabens

Only methylparaben was found in all environmental samples throughout the catchment, though propylparaben was found in 36 of the 56 samples collected. They were found at concentrations between 3.6 to 112.6 ng L^{-1} and <MQL (0.83 ng L^{-1}) to 9.0 ng L^{-1}, respectively. The MQL for ethylparaben and butylparaben are 0.79 and 0.38 ng L^{-1}, respectively. These MECs are similar to those found by Kasprzyk-Hordern et al (UK) and Gorga et al (Spain), with methylparaben at levels between ND (not detected) to 144 ng L^{-1} and ND to 142 ng L^{-1} and propylparaben at ND to 11 ng L^{-1} and ND to 26 ng L^{-1}, though both were detected less frequently than in this study. The ethylparaben was also found in both studies with ND to 15 ng L^{-1} and ND to 49 ng L^{-1} [37, 42]

Of the parabens, butylparaben appears to be the most toxic with a PNEC of 0.006 mg L^{-1}. The remaining parabens decrease in toxicity with their molecular weight, propylparaben (0.025 mg L^{-1}) > ethylparaben (0.210 mg L^{-1}) > methylparaben (2.03 mg L^{-1}). A lower PNEC was determined by De García et al 0.0069 mg L^{-1}, however this was calculated from EC50 data and an AF of 1000, though three trophic levels were considered [43]. The PNEC calculated with acute toxicity data in this study showed a similar PNEC of 0.020 mg L^{-1}, however the more accurate chronic PNEC is used for the RQ calculation.

The chronic $RQ_{MEC:PNEC}$ for methylparaben and propylparaben were far below 0.01 (2.07×10^{-5} and 1.99×10^{-4}). Furthermore, when considered as a class of similarly acting substances they show negligible risk (2.19×10^{-4}). However, more recent studies have begun to show estrogenic effects such as increasing levels of vitellogen production at 0.015 mg L^{-1} of methylparaben when no other lethal or non-lethal effects were seen [44, 45]. The lack of defined EC10 or NOEC data in these studies mean they could not be used in this work. Furthermore, these are effects that may be seen by the other parabens, further work into these effects is required.

3.1.3. Plasticiser – Bisphenol A

The plasticiser bisphenol A was found at 7 of the 8 sampling points. It was detected with increasing frequency (n = 42 across catchment) and concentration down the river, with concentrations ranging from <MQL (0.86 ng L^{-1}) to 116.9 ng L^{-1}. These levels are far lower

than those quantified by Česen et al in Slovenian and Croatian water (44.3 – 2,670 ng L^{-1}) or the highest level, 4,130 ng L^{-1} (Australia), reported in the review by Sousa et al [1, 46, 47]. However, these levels are similar to what has previously been found in the UK [37, 48, 49].
The chronic PNEC for bisphenol A is 0.28 ng L^{-1} this is derived from the experimental data from Oehlmann et al (EC10, 13.9 ng L^{-1}, feminisation of snails) [50]. A previous study has calculated a PNEC of 1000 ng L^{-1}, based on acute toxicity data in algae [51–53], however the endocrine disrupting activity was not taken into account. Furthermore, considering effects on higher trophic levels such as fish, a PNEC of 158 ng L^{-1} can still be calculated (based on EC50 of 158 ug L^{-1} for the vitellogen induction of male fathead minnow and an AF of 1000 due to EC50 and one species).

The chronic $RQ_{MEC:PNEC}$ for BPA based on the lowest PNEC, 0.28 ng L^{-1}, results in high risk for the daphnid trophic levels with $RQ_{MEC:PNEC}$ of 206 at the highest point across the catchment. Sites from R2 to R8 show $RQ_{MEC:PNEC}$s above 1, (ranging from 2.57 at R2 to 207 at R8). When considering the PNEC calculated from the EC50 from the above literature, with a PNEC of 158 ng L^{-1}, the RQ is below 1 (0.364) at its highest across the catchment. Though this shows medium level of risk at current levels, the persistence and increasing bisphenol A levels throughout the catchment suggest that this may become a problem further downstream. This is comparable to levels seen by Riva et al.,[17] who found BPA was also posed a high risk in rivers running through Milan with $RQ_{MEC:PNEC}$ of 0.57 to 1.9.

3.1.4. Steroid estrogens

Steroid estrogens were not found in any of the environmental matrices. This is likely due to the MQLs for these compounds which were 3.92, 4.48, 4.91 ng L^{-1} for E1, E2 and EE2 respectively, which are relatively low compared to many other compounds. However, the PNEC for E1 and E2 are 0.64 and 0.10 ng L^{-1}, which shows this method was not sensitive enough to determine environmental risk for these two estrogens. EE2 however, has a PNEC of 174 ng L^{-1}, which when compared to its MQL shows low risk to the aquatic environment, with a minimum RQ value 0.03. The predicted environmental concentrations at R2 shows potential concentration of 3.03 ng L^{-1} for E1, based on effluent concentration, along with E1s presence in effluent at the other sites shows that E1 is entering the environment and it is likely due to the lack of sensitivity of the method for these compounds and the low population in this catchment compared to other studies (which tend to focus on large cities) that they were unable to be quantified. These CECs are some of the most studied compounds, with environmental concentration determined across 5 continents, reaching 93 ng L^{-1} (Huangpu River, China), 87 ng L^{-1} (Piracicaba River, Brazil), and 1822 ng L^{-1} (Taiwan), for E1, E2 and EE2 respectively [1, 54–56]. These levels in

comparison with the same PNEC calculated in this study shows RQ values greater than 1, (146 for E1, 870 for E2 and 10.5 for EE2). This was comparable to the levels found by Sousa et al [1]. E2 and EE2 are proposed as priority hazardous substances with recommended legislative targets for consent of 0.41 and 0.035 ng L^{-1} annual average EQS, respectively, (0.035 ng L^{-1} based on species sensitivity distribution approach and AF of 2)[57].

Table 1 Acute aquatic ecotoxicity data for three trophic levels with references, assessment factor, notes and PNEC

Class of Analyte	Analyte	Cas no	EC50 Algae mg L-1	Ref	EC50 Crustacean mg L-1	Ref	EC50 Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
UV Filter	Benzophenone-1	131-56-6	2.12	[58]	3.9	[59]	3.7	[58]	1000	Experimental	2700
	Benzophenone-2	131-55-5	2.8	[60]	26.0	[60]	4.5	[60]	1000	Modelled	570.0
	Benzophenone-3	131-57-7	0.67	[58]	1.09	[61]	3.89	[61]	1000	Experimental	162.3
	Benzophenone-4	4065-45-6	25.9	[59]	25.9	[59]	25	[58]	1000	Experimental	135.1
Parabens	Methylparaben	99-76-3	35.25	[62]	11.2	[58]	59.5	[58]	1000	Experimental	2899
	Ethylparaben	120-47-8	37	[58]	7.4	[63]	14	[63]	1000	Experimental	46.00
	Propylparaben	94-13-3	16	[58]	2	[63]	4.9	[63]	1000	Experimental	0.53
	Butylparaben	94-26-8	4	[63]	1.9	[63]	3.1	[63]	1000	Experimental	2678
Plasticizer	Bisphenol A	80-05-7	2.7	[64]	7.8	[65]	5.1	[66]	1000	Experimental	21820
Steroid estrogens	E1	53-16-7	0.57	[58]	1.5	[58]	1.186	[58]	1000	Experimental	56.68
	E2	50-28-2	0.2	[60]	2.87	[65]	0.46	[66]	1000	Experimental	2.00
	EE2	57-63-6	0.1	[60]	1.6	[60]	1.4	[60]	1000	Modelled	40400
Antibiotics and Antibacterial	Sulfasalazine	599-79-1	2.9	[60]	4.6	[60]	11.7	[60]	1000	Modelled	40386
	Clarithromycin	81103-11-9	0.046	[67]	3.3	[60]	5.0	[60]	1000	Modelled	738293
	Azithromycin	83905-01-5	0.00053	[67]	3.0	[60]	18.8	[60]	1000	Modelled	1786232
	Trimethoprim	738-70-5	2.7	[60]	120.7	[68]	100	[68]	1000	Experimental	1240428
	Sulfamethoxazole	723-46-6	21.8	[60]	177.3	[68]	562.5	[68]	1000	Experimental	3301
	Triclosan	3380-34-5	0.1	[60]	0.191	[58]	0.42	[69]	1000	Experimental	N/A
	Amoxicillin	26787-78-0	0.002	[58]	1000	[58]	1000	[58]	1000	Unbounded Experimental	7952
	Metronidazole	443-48-1	40.4	[58]	1000	[58]	100	[58]	1000	Unbounded Experimental	401.4
	Sulfadiazine	68-35-9	40.4	[60]	221	[58]	906.5	[60]	1000	Modelled	140933
	Cefalexin	15686-71-2	879.2	[60]	738.3	[60]	7455.8	[60]	1000	Modelled	143301
	Ofloxacin	82419-36-1	2444.5	[60]	1786.2	[60]	19352.1	[60]	1000	Modelled	3452
	Ciprofloxacin	85721-33-1	1621.6	[60]	1240.4	[60]	13131.4	[60]	1000	Modelled	N/A
	Tetracycline	60-54-8	3.3	[60]	5.3	[60]	78.5	[60]	1000	Modelled	187480
	Danofloxacin	112398-08-0	-	-	-	-	-	-	-	-	49791
	Oxytetracycline	79-57-2	8.0	[60]	11.0	[60]	227.8	[60]	1000	Modelled	110.4
	Chloramphenicol	56-75-7	0.4	[60]	72.1	[60]	38.8	[60]	1000	Modelled	47613
	Penicillin G	61-33-6	140.9	[60]	1221.0	[60]	1226.7	[60]	1000	Modelled	N/A
	Penicillin V	87-08-1	143.3	[60]	1228.1	[60]	1237.8	[60]	1000	Modelled	5633494
	Erythromycin	114-07-8	3.5	[60]	8.6	[60]	15.2	[60]	1000	Modelled	N/A
	Prulifloxacin	123447-62-1	-	-	-	-	-	-	-	-	164452
	Norfloxacin	70458-96-7	2567.5	[60]	187.48	[70]	20081.4	[60]	1000	Modelled	36600
Antifungal	Griseofluvin	126-07-8	102.0	[60]	138.8	[60]	49.8	[60]	1000	Modelled	330.0
	Ketoconazole	65277-42-1	0.2	[60]	1.2	[60]	0.1	[60]	1000	Modelled	90.00
Hypertension	Valsartan	137862-53-4	100	[58]	47.6	[60]	62.8	[60]	1000	Modelled	11850
	Irbesartan	138402-11-6	-	-	-	-	-	-	-	-	4865
	Lisinopril	76547-98-3	9140.4	[60]	5633.5	[60]	66517.8	[60]	1000	Modelled	15.04
	Candesartan Cilexetil	145040-37-5	-	-	-	-	-	-	-	-	4933
NSAIDs	Ketoprofen	22071-15-4	-	-	164.5	[60]	264.1	[60]	1000	Modelled	25280

Class of Analyte	Analyte	Cas no	EC50 Algae mg L-1	Ref	EC50 Crustacean mg L-1	Ref	EC50 Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
	Ibuprofen	15687-27-1	342.2	[71]	36.6	[58]	173	[58]	1000	Experimental	3409268
	Naproxen	22204-53-1	31.82	[72]	0.33	[72]	193.3	[60]	1000	Experimental	1399
	Diclofenac	15307-86-5	71.9	[71]	22.704	[73]	0.09	[74]	1000	Experimental	14300
	Acetaminophen	103-90-2	112.7	[58]	11.85	[58]	160	[68]	1000	Experimental	123.1
Lipid regulator	Bezafibrate	41859-67-0	4.9	[60]	12.2	[60]	17.6	[60]	1000	Modelled	20093
	Atorvastatin	134523-00-5	0.3	[60]	0.3	[60]	0.0	[60]	1000	Modelled	2101
	Gemfibrozil	25812-30-0	10.6	[60]	4.9	[60]	6.7	[60]	1000	Modelled	143000
Antihistamine	Fexofenadine	83799-24-0	25.3	[60]	36.9	[60]	281.9	[60]	1000	Modelled	7300
	Cetirizine	83881-51-0	5127.6	[60]	3409.3	[60]	38741.0	[60]	1000	Modelled	5000
GUD/ED	Sildenafil	139755-83-2	1.4	[60]	5.6	[60]	8.8	[60]	1000	Modelled	8009
Antidiabetics	Metformin	657-24-9	320	[75]	14.3	[76]	1315.5	[76]	1000	Experimental	78001
	Gliclazide	21187-98-4	0.1	[60]	150	[77]	0.9	[60]	1000	Modelled	5542
	Sitagliptin	486460-32-6	20.1	[60]	21.9	[60]	196.8	[60]	1000	Modelled	187.1
Cough suppressant	Pholcodine	509-67-1	2.1	[60]	60.2	[60]	19.5	[60]	1000	Modelled	63.67
Beta-blocker	Atenolol	29122-68-7	143	[78]	755	[78]	1096.4	[60]	1000	Experimental	41100
	Metoprolol	51384-51-1	7.3	[75]	100	[75]	81.6	[60]	1000	Experimental	41755
	Propranolol	525-66-6	5.8	[75]	5	[79]	20.2	[60]	1000	Experimental	12294
	Bisoprolol	66722-44-9	8.0	[60]	9.4	[60]	79.9	[60]	1000	Modelled	202800
H2 receptor agonist	Ranitidine	66357-35-5	95.3	[60]	78.0	[60]	797.9	[60]	1000	Modelled	25.75
	Cimetidine	51481-61-9	5.5	[60]	271.3	[68]	100	[68]	1000	Experimental	6.41
X-ray contrast media	Iopromide	73334-07-3	-	-	0.2	[60]	-	-	1000	Modelled	2290
Various	Buprenorphine	52485-79-7	0.1	[60]	-	-	0.5	[60]	1000	Modelled	897.2
Drug precursor	Ephedrine/pseudoephedrine	299-42-3	41.1	[58]	170.8	[80]	464	[58]	1000	Experimental	9538
	Norephedrine	492-39-7			41.8	[60]	431.0	[60]	1000	Modelled	722.2
Anti-cancer	Azathioprine	446-86-6	12.3	[60]	328.6	[60]	1735.3	[60]	1000	Modelled	N/A
	Methotrexate	59-05-2	202.8	[60]	326.8	[60]	67598.5	[60]	1000	Modelled	34000
	Ifosfamide	3778-73-2	161.2	[60]	0.0	[60]	13.0	[60]	1000	Modelled	9400
	Tamoxifen	10540-29-1	0.0	[60]	0.0	[60]	0.1	[60]	1000	Modelled	27.00
	Imatinib	152459-95-5	2.29	[81]	-	-	565.7	[60]	1000	Modelled	N/A
	Capecitabine	154361-50-9	0.9	[60]	485.8	[60]	-	-	1000	Modelled	43.00
	Bicalutamide	90357-06-5	9.5	[60]	65.5	[60]	70.5	[60]	1000	Modelled	N/A
Anaesthetic and metabolite	Ketamine	6740-88-1	0.7	[60]	1.1	[60]	8.3	[60]	1000	Modelled	1600
	Norketamine	35211-10-0	-	-	-	-	-	-	-	-	N/A
	Venlafaxine	93413-69-5	47.58	[62]	34	[58]	-	-	1000	Experimental	140.0
	Desmethylvenlafaxine	93413-62-8	32.2	[58]	33	[58]	9.4	[58]	1000	Experimental	71.69
	Fluoxetine	54910-89-3	0.027	[82]	0.234	[83]	0.705	[83]	1000	Experimental	43.30
	Norfluoxetine	83891-03-6	-	-	-	-	-	-	-	-	131.9
	Sertraline	79617-96-2	0.043	[82]	0.066	[84]	0.38	[84]	1000	Experimental	40920
	Mirtazapine	85650-52-8	-	-	-	-	-	-	-	-	6250
	Citalopram	59729-33-8	1.6	[82]	20	[82]	4.5	[60]	1000	Experimental	N/A
	Desmethylcitalopram	62498-67-3	-	-	-	-	-	-	-	-	N/A
	Paroxetine	61869-08-7	0.14	[82]	6.24	[85]	3.3	[60]	1000	Experimental	8200
	Duloxetine	116539-59-4	0.1	[60]	0.2	[60]	0.7	[60]	1000	Modelled	90.86
	Amitriptyline	50-48-6	0.0	[60]	4.82	[85]	0.6	[60]	1000	Modelled	61056

Class of Analyte	Analyte	Cas no	EC50 Algae mg L-1	Ref	EC50 Crustacean mg L-1	Ref	EC50 Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
	Nortriptyline	72-69-5	-	-	0.1	[60]	0.8	[60]	1000	Modelled	41553
	Norsertraline	87857-41-8	-	-	-	-	40.9	[60]	1000	Modelled	3545
Anti-epileptic	Carbamazepine	298-46-4	36.6	[86]	76.3	[68]	6.25	[87]	1000	Experimental	1092
	Carbamazepine 10,11-epoxide	36507-30-9	-	-	-	-	-	-	-	-	688.4
	10,11-Dihydro -10-hydroxycarbamazepine	29331-92-8	-	-	-	-	-	-	-	-	68.04
Calcium-channel blocker	Diltiazem	42399-41-7	9.6	[88]	8.2	[68]	15	[68]	1000	Experimental	386.0
	Verapamil	52-53-9	0.1	[60]	21	[89]	0.8	[60]	1000	Modelled	100000
Hypnotic	Temazepam	846-50-4	-	-	61.1	[60]	64.2	[60]	1000	Modelled	350.0
	Oxazepam	604-75-1	-	-	41.6	[60]	44.9	[60]	1000	Modelled	97000
	Diazepam	439-14-5	3.5	[60]	19.8	[60]	22.6	[60]	1000	Modelled	170.0
Anti-psychotic	Quetiapine	111974-69-7	1.1	[60]	1.7	[60]	12.7	[60]	1000	Modelled	13.23
	Risperidone	106266-06-2	0.7	[60]	1.2	[60]	8.3	[60]	1000	Modelled	2892
Dementia	Donepezil	120014-06-4	0.1	[60]	0.2	[60]	1.0	[60]	1000	Modelled	N/A
	Memantine	19982-08-2	0.4	[60]	0.6	[60]	4.6	[60]	1000	Modelled	1331
Lifestyle Chemicals and Creatinine	Creatinine	60-27-5	100	[58]	1000	[58]	2807.0	[60]	1000	Experimental	1231
	Nicotine	54-11-5	37	[58]	0.35	[90]	4.9	[60]	1000	Experimental	1409
	Caffeine	58-08-2	100	[58]	177.49	[70]	97	[58]	1000	Experimental	172.4
	Cotinine	486-56-6	64.5	[60]	0.17	[90]	972.8	[60]	1000	Modelled	N/A
	1,7-dimethylxanthine	611-59-6	0.0	[60]	201.8	[60]	640.5	[60]	1000	Modelled	819.1
Opioid Analgaesics and Metabolites	Heroin	561-27-3	6.6	[60]	11.2	[60]	2.9	[60]	1000	Modelled	954.4
	6-acetylmorphine	2784-73-8	-	-	-	-	-	-	-	-	13018
	Morphine	57-27-2	1.3	[60]	1.3	[60]	12.8	[60]	1000	Modelled	959.3
	Dihydromorphine	509-60-4	2.5	[60]	1.2	[60]	15.1	[60]	1000	Modelled	N/A
	Normorphine	466-97-7	1.5	[60]	1.4	[60]	14.0	[60]	1000	Modelled	N/A
	Methadone	76-99-3	0.2	[60]	0.3	[60]	2.2	[60]	1000	Modelled	2700
	EDDP	30223-73-5	-	-	-	-	-	-	-	-	570.0
	Codeine	76-57-3	0.8	[60]	18.8	[60]	9.2	[60]	1000	Modelled	162.3
	Norcodeine	467-15-2	1.0	[60]	24.1	[60]	10.1	[60]	1000	Modelled	135.1
	Dihydrocodeine	125-28-0	13.0	[60]	14.0	[60]	124.9	[60]	1000	Modelled	2899
	Tramadol	27203-92-5	1.0	[60]	170	[89]	10.2	[60]	1000	Modelled	46.00
	N-desmethyltramadol	75377-45-6	-	-	-	-	-	-	-	-	0.53
	O-desmethyltramadol	144830-15-9/ 144830-14-8	-	-	-	-	-	-	-	-	2678
Stimulants and metabolites	Amphetamine	300-62-9	3.8	[60]	-	-	37.6	[60]	1000	Modelled	3803
	Methamphetamine	537-46-2	2.0	[60]	2.5	[60]	20.5	[60]	1000	Modelled	1967
	MDMA	42542-10-9	2.3	[60]	0.2	[60]	24.2	[60]	1000	Modelled	215.8
	MDA	101-77-9	4.7	[60]	0.35	[58]	20.6	[58]	1000	Modelled	350.0
	Cocaine	50-36-2	4.4	[60]	5.5	[60]	32.3	[60]	1000	Modelled	4350
	Benzoylecgonine	519-09-5	12041.7	[60]	6805.2	[60]	33458.8	[60]	1000	Modelled	6805166
	Anhydroecgoninemethylester	43021-26-7	-	-	-	-	2.7	[60]	1000	Modelled	2675
	Cocaethylene	529-38-4	-	-	-	-	-	-	-	-	N/A

Class of Analyte	Analyte	Cas no	EC50 Algae mg L-1	Ref	EC50 Crustacean mg L-1	Ref	EC50 Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
	Mephedrone	1189805-46-6	-	-	-	-	-	-	-	-	N/A
	MDPV	687603-66-3	0.2	[60]	0.3	[60]	-	-	1000	Modelled	210.2
Pesticides, fungicides and herbicides	Thiamethoxam	153719-23-4	81.8	[58]	0.014	[58]	100	[58]	1000	Experimental	14.00
	Imidacloprid	138261-41-3	10	[58]	85	[58]	211	[58]	1000	Experimental	10000
	Clothiniadin	210880-92-5	42.8	[60]	37.9	[60]	354.2	[60]	1000	Modelled	37941
	Metazachlor	67129-08-2	0.0	[60]	10.8	[60]	5.2	[60]	1000	Modelled	21.89
	Terbuthylazine	5915-41-3	0.1	[60]	8.8	[60]	9.0	[60]	1000	Modelled	63.58
	Methiocarb	2032-65-7	1.4	[60]	0.0	[60]	2.8	[60]	1000	Modelled	3.09
	Dichlofluanid	1085-98-9	36.9	[60]	37.6	[60]	62.0	[60]	1000	Modelled	36867
	Flufenacet	142459-58-3	7.0	[60]	45.7	[60]	49.9	[60]	1000	Modelled	7000
	Oxadiazon	19666-30-9	0.2	[60]	0.4	[60]	0.1	[60]	1000	Modelled	75.08
	Chlorpyrifos	2921-88-2	0.3	[60]	0.0	[60]	0.1	[60]	1000	Modelled	0.33
	Triallate	2303-17-5	0.1	[60]	0.3	[60]	0.3	[60]	1000	Modelled	126.3
Veterinary Pharmaceuticals	Tylosin	1401-69-0	0.21	[67]	6.2	[60]	-	-	1000	Modelled	210.0
	Sulfapyridine	144-83-2	5.28	[58]	-	-	246.3	[60]	1000	Modelled	5280
	Sarafloxacin	98105-99-8	329.8	[60]	322.8	[60]	3016.7	[60]	1000	Modelled	322803
	Ceftiofur	80370-57-6	114.4	[60]	69.8	[60]	53.3	[60]	1000	Modelled	53271
	Diazinon	333-41-5	1.4	[60]	0.0	[60]	7.97	[91]	1000	Modelled	1.23

3.1.5. Antibiotics and antibacterials

Of the 21 antibiotics and antibacterials in this method, 12 were found in the environment, 3 were found in all environmental samples (sulfasalazine 10.4 – 61.9 ng L^{-1}, clarithromycin 39.8 – 178.8 ng L^{-1}, and sulfamethoxazole 26.1 – 51.3 ng L^{-1}). The highest concentration found was erythromycin at 2,148 ng L^{-1}, though this value may be exaggerated due to correction for poor recovery (uncorrected values were 140.7 ng L^{-1} on average). The macrolide antibiotics azithromycin, clarithromycin and erythromycin are in the 17 CECs prioritised by Directive 2015/495/EU. In this study, concentrations for azithromycin and erythromycin ranged from <MQL (0.26 ng L^{-1}) to 21.9 ng L^{-1} and <MQL (3.83 ng L^{-1}) to 2,410 ng L^{-1} respectively. In literature they have been found at concentrations up to 16,633 ng L^{-1}, 2,403 ng L^{-1}, 65.1 ng L^{-1} (for azithromycin, clarithromycin and erythromycin) in El Albujón watercourse in Spain [92]. These were identified as the highest concentrations for azithromycin and clarithromycin in literature since 2012 in a review by Sousa et al [1]. Other antibiotics of note include sulfamethoxazole, identified as one of the most reported antibiotics, excluding Watch List antibiotics, by Sousa et al, and reported up to 1,820 ng L^{-1} (Taiwan), [1, 54]. In this study it was found at concentrations up to 44.7 ng L^{-1}, similar to levels seen in the El Albujón watercourse in Spain.

PNECs ranged from 0.12 mg L^{-1} for norfloxacin to 10 ng L^{-1} for amoxicillin (based on chronic data for daphnid trophic level from ECHA database (1.0 µg L^{-1}, AF of 100)). Of the PNEC detected in the environment in this study concentrations ranged from 62 ng L^{-1} for azithromycin to 50,640 ng L^{-1} for cefalexin. PNECs were previously calculated for several antibiotics (n = 28) by Verlicchi et al [93], ranging from 3.7 ng L^{-1} for amoxicillin to 938,000 ng L^{-1} for ciprofloxacin but this was based on acute effects (EC50). PNECs could not be calculated for danofloxacin or prulifloxacin as no ecotoxicity data could be found, nor could it be predicted using ECOSAR. The $RQ_{MEC:PNEC}$ ranged from 0.002 for penicillin V to 2.875 for erythromycin. Several antibiotics were in the range of 0.204 – 0.549. Ranked for risk to the environment from least to most toxic, penicillin V (0.002) < sulfadiazine (0.004) < cefalexin (0.005) < metronidazole (0.021) < sulfamethoxazole (0.068) < ciprofloxacin (0.204) < sulfasalazine (0.267) < azithromycin (0.270) < chloramphenicol (0.355) < clarithromycin (0.379) < trimethoprim (0.549) < erythromycin (2.875). Sulfamethoxazole has previously been classified as medium risk with RQ of 0.1-1 and erythromycin and ciprofloxacin were classed as high risk with RQ < 1 by Sousa et al [1].

The $RQ_{mixture}$ is above 1 at 6 out of 8 sampling sites, (0.51 at R1, 0.791 at R3, both upstream of WwTW discharges). There is an increasing trend for $RQ_{mixture}$ downstream ranging from 0.51

at R1 to 2.12 at R8, with a spike of 3.99 at R4. Sum of toxic units shows the algal trophic layer are most at risk.

One aspect this approach does not consider is the potential development of antibiotics resistance at the low levels found within the environment. This is a crucial consideration for future work with regards to antibiotics.

3.1.6. Antifungals

Of the two antifungals in this study, only ketoconazole was found in the environment. It was found in 19 out of 56 samples taken, but it was found at least once at each site, at concentrations ranging from 3.1 to 93.8 ng L^{-1}. Ketoconazole has been found in Swedish coastal waters <MQL (<0.1 ng L^{-1}) at 0.22 ng L^{-1} [20]. Another study found ketoconazole at low level, between 200 m upstream of an effluent discharge point and several sites downstream up to 3 km away, at concentrations ranging from 2.7-3.7 ng L^{-1} in the aqueous phase [14]. However, two other studies have not found this antifungal in environmental waters [94, 95].

With regards to ecotoxicity, ketoconazole is the more toxic of the two antifungals, with chronic PNEC of 20.3 ng L^{-1}, compared to 2,990 ng L^{-1} for griseofulvin. For the concentrations throughout the catchment, ketoconazole presents a high risk to the catchment, with $RQ_{MEC:PNEC}$ values ranging from 3.51 at R1 to 2.0 at R8. Interestingly, this is the reverse distribution trend than for many compounds, which generally show low-to-high levels from R1 to R8. This suggests the main source of ketoconazole is not from the WwTWs of the catchment and is from a source higher upstream than the sampled area. Overall average RQ for this stretch of the catchment river is 3.5.

Similarly, to antibiotics, low levels of antifungals in the environment can encourage the spread of antifungal resistance. These sublethal effects can have important implication for the future of medicine.

3.1.7. Hypertension

Of the four pharmaceuticals for treating hypertension of this study, lisinopril and candesartan were not found in the environment (<MQL = 21.7 ng L^{-1} and 23.0 ng L^{-1} respectively), valsartan was found in 14 samples, at the lower end of the catchment (R6-R8) at average concentrations per site from 55.0 to 64.0 ng L^{-1} (<MQL = 9.26 ng L^{-1}). Concentrations at R2 were calculated as 9.66 ng L^{-1} and irbesartan was found in all samples at concentrations ranging from 21.3 to

134.4 ng L^{-1}. Valsartan was found at similar concentration in the UK <MQL (<0.5) to 73 ng L^{-1} [37], and concentrations up to 7,479 ng L^{-1} were found in El Albujón watercourse (Spain), in another one-week intensive study at the same watercourse, average concentrations depended on season and varied from 18.5 ± 12.2 ng L^{-1} in autumn (Mean ± SD) to 466.9 ± 347.6 ng L^{-1} in summer [92]. Concentrations for irbesartan were found up to 305.5 ng L^{-1} in the El Albujón watercourse (Spain), seasonal trends shows 152.4 ± 150.6 ng L^{-1} in summer to 71.8 ± 65.5 ng L^{-1} in winter, undetectable during sampling campaigns in other seasons [92]. To this author's knowledge lisinopril has not yet been quantified in the aqueous environment.

The ecotoxicity of irbesartan is largely unknown with no data found in TOXNET or ECOTOX database, no modelled data available through ECOSAR or any literature found in searches of 'irbesartan' AND 'toxicity' AND 'algae' OR 'daphnia' OR 'crustacean' OR 'fish'. Valsartan is the more ecotoxic than lisinopril, with a PNEC of 0.14 mg L^{-1} compared to 0.33 mg L^{-1}. The highest $RQ_{MEC:PNEC}$ for the catchment for valsartan is very low, 4.4 x 10^{-4}, showing negligible risk for this compound. Assuming irbesartan would exhibit similar ecotoxicity as seen by valsartan, the highest $RQ_{MEC:PNEC}$ is 7.7 x 10^{-3}. Overall, this suggests a low risk to the aquatic environment from these compounds.

3.1.8. NSAIDs and acetaminophen

Of the 4 NSAIDs and acetaminophen, 4 are found in the environment. Only ketoprofen was not found. The rest were found in all samples collected. Ibuprofen, naproxen, diclofenac and acetaminophen were found at the concentration ranges 8.3 to 159.2 ng L^{-1}, 85.2 to 462.4 ng L^{-1}, 11.0 to 98.9 ng L^{-1}, and 13.2 to 488.3 ng L^{-1} respectively. Diclofenac in particular is part of the Watch List as a prospective priority hazardous substance with recommended average annual EQS of 100 ng L^{-1} [57]. Within this study diclofenac does not quite reach this level, but is within 20% of this limit. Compared to another study in the UK, this catchment appears to show a wider range of concentrations that previously seen for all compounds except acetaminophen, which range from 185 to 1534 ng L^{-1} in a 5 month monitoring program in 2009 [49]. The highest concentration for diclofenac in literature since 2012, was 7,761 ng L^{-1}, based on the review by Sousa et al [1], was found in the Antarctic Peninsula [96]. The other NSAIDs, showed similar high level concentrations in the µg L^{-1} range, ketoprofen 9,220 ng L^{-1} and naproxen 59,300 ng L^{-1} these were found in South African surface water. Ibuprofen showed lower levels in this environment with 2,570 ng L^{-1}. Levels for acetaminophen have reached 30,421 ng L^{-1} during a 3 years study in Monjolinho River (Brazil) [97].

Ketoprofen and acetaminophen have been shown to be similarly toxic, with PNEC of 0.021 mg L^{-1} although, acetaminophen is based on chronic experimental data with an applied AF of 10, compared to ketoprofens modelled data and applied AF of 1000. Therefore, the ecotoxicity ranking is acetaminophen ≈ ketoprofen > naproxen (0.426 mg L^{-1}) > diclofenac (0.464 mg L^{-1}) > ibuprofen (1.0 mg L^{-1}). The highest $RQ_{MEC:PNEC}$ is for acetaminophen with 0.013, showing there is limited risk to the environment individually. Furthermore, the $RQ_{mixture}$ shows only slightly more risk than acetaminophen itself. These five pharmaceuticals were also considered by Riva et al., and they shows a similar lack of risk in the surface water of Milan also [17].

3.1.9. Lipid Regulators

Bezafibrate was the most frequently found lipid regulator in this study. With concentrations of 29.4 to 131.4 ng L^{-1} across all 56 samples collected. Atorvastatin, however, was only found in 42 samples (it was not found in any samples upstream of WwTW A (R1), with concentrations ranging from 9.1 to 57.5 ng L^{-1}. Gemfibrozil was only found in 3 samples at concentrations ranging from 1.5 to 37.3 ng L^{-1}.

Bezafibrate and gemfibrozil are frequently monitored in the environment, despite not being present on a watch list, at concentrations up to approximately 950 and 850 ng L^{-1} respectively [1]. Atorvastatin and its metabolites para- and ortho-hydroxylatorvastatin have been identified as particularly hazardous to the environment and have been included in the second stage of the UKWIR CIP (CIP2) programme for further monitoring in the UK [98].

Of the three atorvastatin poses the greatest risk to the aquatic environment with $RQ_{MEC:PNEC}$ >1 at six of the sites across the catchment (R2, R3, R4, R6, R7, R8). Furthermore, this does not take into account the risk from the metabolites of this compound which are likely to be present. The risk posed by bezafibrate is an order of magnitude lower than atorvastatin, but would be classed as medium risk at six of the sites (between $RQ_{MEC:PNEC}$ is 0.1-1 for sites R3-R8, by the RQ assessment by Sousa [1]).

Table 2 Chronic aquatic ecotoxicity data for three trophic levels with references, assessment factor, notes and PNEC

Class of Analyte	Analyte	Cas no	EC10 /NOEC Algae mg L-1	Ref	EC10 /NOEC Crustacean mg L-1	Ref	EC10 /NOEC Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
UV Filters	Benzophenone-1	131-56-6	0.33	[58]	5.77	[58]	1.45	[58]	10	Experimental	32700
	Benzophenone-2	131-55-5	0.42	[60]	9.27	[60]	2.21	[60]	1000	Modelled	421.8
	Benzophenone-3	131-57-7	0.18	[58]	0.19	[99]	0.72	[58]	10	Experimental	18000
	Benzophenone-4	4065-45-6	664.23	[60]	5.00	[58]	4.90	[58]	50	Experimental	97940
Parabens	Methylparaben	99-76-3	20.34	[62]	27.01	[62]	25.00	[100]	10	Experimental	2034000
	Ethylparaben	120-47-8	2.10	[58]	2.30	[100]	17.00	[100]	10	Experimental	210000
	Propylparaben	94-13-3	2.10	[58]	0.25	[58]	2.50	[100]	10	Experimental	25000
	Butylparaben	94-26-8	0.80	[58]	0.31	[60]	1.00	[100]	50	Experimental	6146
Plasticizer	Bisphenol A	80-05-7	0.23	[60]	1.39E-05	[50]	7.77	[91]	50	Experimental	0.28
Steroid estrogens	E1	53-16-7	0.57	[58]	0.44	[60]	0.00	[101]	50	Experimental	0.64
	E2	50-28-2	3.10	[58]	138.70	[58]	0.00	[58]	10	Experimental	0.10
	EE2	57-63-6	0.47	[60]	0.25	[60]	0.17	[60]	1000	Modelled	174.1
Antibiotics and Antibacterial	Sulfasalazine	599-79-1	0.72	[60]	0.82	[60]	0.20	[60]	1000	Modelled	200.9
	Clarithromycin	81103-11-9	0.00	[99]	5.00	[99]	68.00	[99]	10	Experimental	370.0
	Azithromycin	83905-01-5	0.01	[99]	0.29	[60]	0.82	[60]	100	Experimental	62.00
	Trimethoprim	738-70-5	0.77	[60]	0.08	[60]	3.59	[60]	1000	Modelled	80.74
	Sulfamethoxazole	723-46-6	0.07	[99]	125.06	[60]	5.00	[60]	100	Experimental	660.0
	Triclosan	3380-34-5	0.00	[99]	0.21	[62]	0.04	[99]	10	Experimental	54.00
	Amoxicillin	26787-78-0	5.12	[60]	0.00	[58]	1.73	[60]	100	Experimental	10.00
	Metronidazole	443-48-1	2.70	[60]	3.08	[60]	0.95	[60]	1000	Modelled	949.2
	Sulfadiazine	68-35-9	28.99	[60]	452.78	[60]	21.84	[60]	1000	Modelled	21837
	Cefalexin	15686-71-2	255.35	[60]	50.64	[60]	110.61	[60]	1000	Modelled	50640
	Ofloxacin	82419-36-1	1.30	[99]	0.03	[99]	2456.84	[60]	50	Experimental	580.0
	Ciprofloxacin	85721-33-1	455.22	[60]	81.27	[60]	1553.58	[60]	1000	Modelled	81270
	Tetracycline	60-54-8	0.79	[60]	1.25	[60]	1.29	[60]	1000	Modelled	789.0
	Danofloxacin	112398-08-0	-	-	-	-	-	-	-	-	N/A
	Oxytetracycline	79-57-2	1.69	[60]	2.84	[60]	4.48	[60]	1000	Modelled	1688
	Chloramphenicol	56-75-7	0.22	[60]	47.61	[60]	15.46	[60]	1000	Modelled	217.1
	Penicillin G	61-33-6	84.12	[60]	191.19	[60]	14.99	[60]	1000	Modelled	14988
	Penicillin V	87-08-1	86.33	[60]	193.80	[60]	15.26	[60]	1000	Modelled	15263
	Erythromycin	114-07-8	2.20	[60]	0.75	[60]	1.98	[60]	1000	Modelled	747.2
	Prulifloxacin	123447-62-1	-	-	-	-	-	-	-	-	N/A
	Norfloxacin	70458-96-7	702.54	[60]	115.88	[60]	2647.20	[60]	1000	Modelled	115882
Antifungal	Griseofluvin	126-07-8	50.78	[60]	13.80	[60]	2.99	[60]	1000	Modelled	2990
	Ketoconazole	65277-42-1	0.11	[60]	0.02	[60]	0.03	[60]	1000	Modelled	20.31
Hypertension	Valsartan	137862-53-4	100.00	[58]	18.40	[60]	14.46	[60]	100	Experimental	144635
	Irbesartan	138402-11-6	-	-	-	-	-	-	-	-	N/A
	Lisinopril	76547-98-3	1614.70	[60]	331.92	[60]	801.46	[60]	1000	Modelled	331920
	Candesartan Cilexetil	145040-37-5	-	-	-	-	-	-	-	-	N/A
NSAIDs	Ketoprofen	22071-15-4	57.73	[60]	20.74	[60]	28.78	[60]	1000	Modelled	20737
	Ibuprofen	15687-27-1	11.00	[99]	66.00	[71]	10.00	[58]	10	Experimental	1000000
	Naproxen	22204-53-1	321.50	[71]	39.50	[71]	21.31	[60]	50	Experimental	426199

Class of Analyte	Analyte	Cas no	EC10 /NOEC Algae mg L-1	Ref	EC10 /NOEC Crustacean mg L-1	Ref	EC10 /NOEC Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
	Diclofenac	15307-86-5	49.20	[71]	15.20	[71]	4.64	[91]	10	Experimental	464000
	Acetaminophen	103-90-2	490.00	[99]	0.21	[99]	95.00	[58]	10	Experimental	21000
Lipid regulator	Bezafibrate	41859-67-0	8.40	[60]	4.62	[60]	0.62	[60]	1000	Modelled	618.4
	Atorvastatin	134523-00-5	0.19	[60]	0.02	[60]	0.05	[60]	1000	Modelled	15.16
	Gemfibrozil	25812-30-0	4.89	[60]	0.98	[60]	0.89	[60]	1000	Modelled	889.0
Antihistamine	Fexofenadine	83799-24-0	8.98	[60]	3.33	[60]	12.31	[60]	1000	Modelled	3326
	Cetirizine	83881-51-0	1367.74	[60]	208.50	[60]	5688.08	[60]	1000	Modelled	208500
GUD/ED	Sildenafil	139755-83-2	0.68	[60]	0.28	[60]	0.21	[60]	1000	Modelled	207.1
Antidiabetics	Metformin	657-24-9	1040.59	[60]	4.40	[76]	8360.70	[60]	100	Experimental	44000
	Gliclazide	21187-98-4	0.02	[60]	0.38	[60]	1.17	[60]	1000	Modelled	20.40
	Sitagliptin	486460-32-6	5.16	[60]	1.68	[60]	0.73	[60]	1000	Modelled	728.4
Cough suppressant	Pholcodine	509-67-1	16.14	[60]	4.60	[60]	18.03	[60]	1000	Modelled	4597
Beta-blocker	Atenolol	29122-68-7	27.00	[99]	7.70	[99]	15.22	[60]	50	Experimental	154000
	Metoprolol	51384-51-1	2.69	[60]	0.74	[60]	5.29	[60]	1000	Modelled	744.6
	Propranolol	525-66-6	0.10	[99]	0.76	[99]	0.22	[99]	10	Experimental	10000
	Bisoprolol	66722-44-9	2.62	[60]	0.75	[60]	4.93	[60]	1000	Modelled	754.2
H2 receptor agonist	Ranitidine	66357-35-5	27.42	[60]	5.28	[60]	85.04	[60]	1000	Modelled	5284
	Cimetidine	51481-61-9	2.28	[60]	2.04	[60]	0.77	[60]	1000	Modelled	773.8
X-ray contrast media	Iopromide	73334-07-3	-	-	-	-	-	-	-	-	N/A
Various	Buprenorphine	52485-79-7	0.03	[60]	0.06	[60]	0.01	[60]	1000	Modelled	10.04
Drug precursor	Ephedrine/pseudoephedrine	299-42-3	3.44	[58]	1.69	[60]	21.61	[60]	100	Experimental	16850
	Norephedrine	492-39-7	14.84	[60]	2.80	[60]	47.20	[60]	1000	Modelled	2804
Anti-cancer	Azathioprine	446-86-6	4.76	[60]	5.63	[60]	1.68	[60]	1000	Modelled	1682
	Methotrexate	59-05-2	45.34	[60]	2.93	[60]	1420.87	[60]	1000	Modelled	2933
	Ifosfamide	3778-73-2	29.62	[60]	295.75	[60]	0.02	[60]	1000	Modelled	16.80
	Tamoxifen	10540-29-1	0.00	[60]	0.00	[60]	0.00	[60]	1000	Modelled	1.35
	Imatinib	152459-95-5	0.79	[81]	-	-	56.86	[60]	100	Experimental	7900
	Capecitabine	154361-50-9	-	-	52.33	[60]	-	-	1000	Modelled	52334
	Bicalutamide	90357-06-5	6.94	[60]	12.11	[60]	1.05	[60]	1000	Modelled	1049
Anaesthetic and metabolite	Ketamine	6740-88-1	0.26	[60]	0.11	[60]	0.33	[60]	1000	Modelled	105.7
	Norketamine	35211-10-0	-	-	-	-	-	-	-	-	N/A
	Venlafaxine	93413-69-5	10.00	[62]	68.00	[62]	0.28	[60]	10	Experimental	28398
	Desmethylvenlafaxine	93413-62-8	0.10	[60]	8.20	[58]	2.10	[58]	50	Experimental	2098
	Fluoxetine	54910-89-3	31.34	[102]	170.00	[103]	9.00	[103]	10	Experimental	900000
	Norfluoxetine	83891-03-6	-	-	-	-	-	-	-	-	N/A
	Sertraline	79617-96-2	4.57	[102]	0.03	[84]	0.10	[84]	10	Experimental	3200
	Mirtazapine	85650-52-8	-	-	-	-	-	-	-	-	N/A
	Citalopram	59729-33-8	0.14	[60]	0.07	[60]	0.14	[60]	1000	Modelled	65.23
	Desmethylcitalopram	62498-67-3	-	-	-	-	-	-	-	-	N/A
	Paroxetine	61869-08-7	0.10	[60]	0.05	[60]	0.10	[60]	1000	Modelled	50.48
	Duloxetine	116539-59-4	0.03	[60]	0.02	[60]	0.02	[60]	1000	Modelled	17.91
	Amitriptyline	50-48-6	0.02	[60]	0.01	[60]	0.01	[60]	1000	Modelled	11.86
	Nortriptyline	72-69-5	0.02	[60]	0.01	[60]	0.02	[60]	1000	Modelled	14.76

Class of Analyte	Analyte	Cas no	EC10 /NOEC Algae mg L-1	Ref	EC10 /NOEC Crustacean mg L-1	Ref	EC10 /NOEC Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
	Norsertraline	87857-41-8	-	-	-	-	-	-	-	-	N/A
Anti-epileptic	Carbamazepine	298-46-4	0.60	[62]	0.03	[73]	1.03	[91]	10	Experimental	2500
	Carbamazepine 10,11-epoxide	36507-30-9	-	-	-	-	-	-	-	-	N/A
	10,11-Dihydro -10-hydroxycarbamazepine	29331-92-8	-	-	-	-	-	-	-	-	N/A
Calcium-channel blocker	Diltiazem	42399-41-7	0.76	[60]	0.28	[60]	0.51	[60]	1000	Modelled	281.2
	Verapamil	52-53-9	0.04	[60]	11.00	[89]	0.03	[60]	100	Experimental	274.4
Hypnotic	Temazepam	846-50-4	5.61	[60]	10.68	[60]	0.90	[60]	1000	Modelled	895.3
	Oxazepam	604-75-1	4.51	[60]	7.75	[60]	0.67	[60]	1000	Modelled	674.8
	Diazepam	439-14-5	3.08	[60]	4.24	[60]	0.40	[60]	1000	Modelled	401.6
Anti-psychotic	Quetiapine	111974-69-7	0.40	[60]	0.16	[60]	0.49	[60]	1000	Modelled	162.1
	Risperidone	106266-06-2	0.26	[60]	0.11	[60]	0.28	[60]	1000	Modelled	114.2
Dementia	Donepezil	120014-06-4	0.03	[60]	0.02	[60]	0.02	[60]	1000	Modelled	18.09
	Memantine	19982-08-2	0.14	[60]	0.06	[60]	0.17	[60]	1000	Modelled	60.76
Lifestyle Chemicals and Creatinine	Creatinine	60-27-5	59.07	[60]	13.15	[60]	32.42	[60]	1000	Modelled	13155
	Nicotine	54-11-5	5.20	[58]	0.02	[58]	3.00	[58]	10	Experimental	2000
	Caffeine	58-08-2	120.00	[99]	35.00	[99]	31.00	[99]	10	Experimental	3100000
	Cotinine	486-56-6	19.76	[60]	109.33	[60]	6.13	[60]	1000	Modelled	6129
	1,7-dimethylxanthine	611-59-6	0.00	[60]	5.55	[60]	1.49	[60]	1000	Modelled	3.67
Opioid Analgaesics and Metabolites	Heroin	561-27-3	1.40	[60]	-	-	0.06	[60]	1000	Modelled	63.85
	6-acetylmorphine	2784-73-8	-	-	-	-	-	-	-	-	N/A
	Morphine	57-27-2	0.48	[60]	0.28	[60]	0.17	[60]	1000	Modelled	165.1
	Dihydromorphine	509-60-4	0.42	[60]	0.26	[60]	0.14	[60]	1000	Modelled	140.4
	Normorphine	466-97-7	0.53	[60]	0.30	[60]	0.19	[60]	1000	Modelled	185.4
	Methadone	76-99-3	0.07	[60]	0.04	[60]	0.06	[60]	1000	Modelled	36.09
	EDDP	30223-73-5	-	-	-	-	-	-	-	-	N/A
	Codeine	76-57-3	5.73	[60]	1.43	[60]	4.79	[60]	1000	Modelled	1425
	Norcodeine	467-15-2	7.05	[60]	1.78	[60]	6.28	[60]	1000	Modelled	1781
	Dihydrocodeine	125-28-0	4.14	[60]	1.09	[60]	8.71	[60]	1000	Modelled	1089
	Tramadol	27203-92-5	0.35	[60]	52.00	[89]	0.45	[60]	100	Experimental	3466
	N-desmethyltramadol	75377-45-6	-	-	-	-	-	-	-	-	N/A
	O-desmethyltramadol	144830-15-9/ 144830-14-8	-	-	-	-	-	-	-	-	N/A
Stimulants and metabolites	Amphetamine	300-62-9	1.24	[60]	0.35	[60]	2.39	[60]	1000	Modelled	348.1
	Methamphetamine	537-46-2	0.67	[60]	0.21	[60]	1.10	[60]	1000	Modelled	211.3
	MDMA	42542-10-9	0.78	[60]	0.22	[60]	0.86	[60]	1000	Modelled	222.8
	MDA	101-77-9	1.18	[60]	0.01	[58]	0.15	[60]	100	Experimental	50.00
	Cocaine	50-36-2	1.46	[60]	0.46	[60]	2.34	[60]	1000	Modelled	459.1
	Benzoylecgonine	519-09-5	3027.30	[60]	384.22	[60]	5050.13	[60]	1000	Modelled	384223
	Anhydroecgoninemethylester	43021-26-7	-	-	0.04	[60]	-	-	1000	Modelled	41.18
	Cocaethylene	529-38-4	-	-	-	-	-	-	-	-	N/A

Class of Analyte	Analyte	Cas no	EC10 /NOEC Algae mg L-1	Ref	EC10 /NOEC Crustacean mg L-1	Ref	EC10 /NOEC Fish mg L-1	Ref	AF	Notes	PNEC ng L-1
	Mephedrone	1189805-46-6	-	-	-	-	-	-	-	-	N/A
	MDPV	687603-66-3	0.08	[60]	-	-	0.08	[60]	1000	Modelled	77.40
Pesticides, fungicides and herbicides	Thiamethoxam	153719-23-4	3.56	[60]	5.74	[60]	20.00	[58]	100	Experimental	35576
	Imidacloprid	138261-41-3	10.00	[58]	1.80	[58]	50.00	[58]	10	Experimental	180000
	Clothiniadin	210880-92-5	3.48	[60]	2.67	[60]	35.07	[60]	1000	Modelled	2674
	Metazachlor	67129-08-2	0.00	[60]	0.19	[60]	0.13	[60]	1000	Modelled	4.93
	Terbuthylazine	5915-41-3	0.32	[60]	0.47	[60]	0.67	[60]	1000	Modelled	318.0
	Methiocarb	2032-65-7	0.31	[60]	0.01	[60]	0.20	[60]	1000	Modelled	6.15
	Dichlofluanid	1085-98-9	11.19	[60]	4.41	[60]	6.55	[60]	1000	Modelled	4413
	Flufenacet	142459-58-3	5.32	[60]	8.76	[60]	0.78	[60]	1000	Modelled	775.2
	Oxadiazon	19666-30-9	0.04	[60]	0.03	[60]	0.04	[60]	1000	Modelled	26.51
	Chlorpyrifos	2921-88-2	0.25	[60]	0.00	[60]	0.00	[60]	1000	Modelled	0.05
	Triallate	2303-17-5	0.22	[60]	0.02	[60]	0.02	[60]	1000	Modelled	18.37
Veterinary Pharmaceuticals	Tylosin	1401-69-0	0.06	[67]	-	-	-	-	100	Experimental	640.0
	Sulfapyridine	144-83-2	10.43	[60]	0.07	[60]	4.54	[60]	1000	Modelled	65.75
	Sarafloxacin	98105-99-8	101.28	[60]	23.87	[60]	244.10	[60]	1000	Modelled	23875
	Ceftiofur	80370-57-6	63.74	[60]	0.82	[60]	1.11	[60]	1000	Modelled	821.1
	Diazinon	333-41-5	1.00	[60]	0.00	[60]	3.33	[91]	100	Experimental	0.80

3.1.10 Further Class Observations

3.1.10.1 Antihistamines

Both fexofenadine and cetirizine were found in all environmental samples at concentrations from 89.0 to 574.9 ng L^{-1} and 44.3 to 585.5 ng L^{-1} respectively. Fexofenadine is far more toxic than cetirizine, leading to a PNEC two orders of magnitude lower than cetirizine, 3 µg L^{-1} to 208 µg L^{-1}. Overall, these compounds pose low risk to the environment, however the apparent persistence and increasing levels of these CECs through the catchment show an increasing risk to the environment with an $RQ_{MEC:PNEC}$ up to 0.136, fexofenadine is of medium risk to the aquatic environment towards lower end of the catchment. Cetirizine contribute <2% to the $RQ_{mixture}$ for this class.

3.1.10.2 GUD/ED

Sildenafil was found in 39 out of the 56 samples collected. Concentrations ranged from 0.1 to 3.5 ng L^{-1}. PNEC was calculated as 207 ng L^{-1}, showing this compound poses no immediate threat to this catchment with an $RQ_{MEC:PNEC}$ of less than 0.01 at most locations.

3.1.10.3 Antidiabetics

All three antidiabetics of this study were found throughout the environment. Metformin was found in 49 samples at concentrations ranging from 100.7 to 5,189.5 ng L^{-1}. Gliclazide was the most ubiquitous and found in all samples, but at the lowest range of concentrations, from 6.4 to 60.1 ng L^{-1}. Sitagliptin was found in 55 samples and at concentrations ranging from 58.3 to 229.1 ng L^{-1}. Despite being present at the highest concentrations metformin poses the least threat to the environment with a PNEC of 44 µg L^{-1} providing an $RQ_{MEC:PNEC}$ of 0.093 at the highest point of the catchment. Sitagliptin poses a medium risk to the aquatic environment with $RQ_{MEC:PNEC}$ of 0.275. However, gliclazide, with the lowest concentrations of the three is of high risk to the environment with $RQ_{MEC:PNEC}$ >1 at out of 8 sites across the catchment and up to 2.321 at R4. The $RQ_{mixture}$ was 2.598 at the highest (R4). The STU showed that the algal trophic layer were most at risk though the RQ_{STU} was below 0.01.

3.1.10.4 Cough Suppressant

The cough suppressant pholcodine was not found in the environment, with a PNEC of 4,596 ng L^{-1} and MQL of 7.42 ng L^{-1}, this compound appears to be of low to no risk to the aquatic environment within this catchment.

3.1.10.5 Beta-blockers

Of the beta blockers in the study atenolol and propranolol were found most frequently and in all environmental samples. Metoprolol and bisoprolol were only found in at site R2, which were predicted from calculation based on effluent concentrations, measured upstream concentrations and flow. (<MQL 0.35, 0.004 ng L^{-1}, respectively). Atenolol and propranolol are the least toxic with PNECs of 154 µg L^{-1} to 10 µg L^{-1}. No $RQ_{MEC:PNEC}$ for these compounds are above > 0.002 and therefore show little risk either individually or together.

3.1.10.6 H2 Receptor agonist

Ranitidine was only found in 49 out of 56 environmental samples at concentrations ranging from 52.3 to 246.3 ng L^{-1}, with a chronic PNEC of 5,283 ng L^{-1}, this shows $RQ_{MEC:PNEC}$ of 0.03 at the highest point in the catchment showing low risk to the aquatic environment. Cimetidine was not found in any samples (<MQL 7.98 ng L^{-1}). At the level of MQL this compounds poses no risk with $RQ_{mixture}$ of >0.01. Overall this class poses little risk to the environment.

3.1.10.7 X-ray contrast media

Iopromide was not found in the environment above MQL (29.9 ng L^{-1}). The PNEC for this compound is 187 ng L^{-1} (based on EC50) which means that iopromide may pose a low risk to the aquatic environment at unmeasurable levels (<29.9 ng L^{-1}) with $RQ_{MEC:PNEC}$ of 0.160.

3.1.10.8 Buprenorphine

Buprenorphine was found in 19 samples out of 56 and was found at all sites except for R5, at concentration ranges from 0.7 to 11.9 ng L^{-1}. The compound has a PNEC of 10.0 ng L^{-1} showing that this compound is at high aquatic risk at the environmental concentrations. The highest concentrations were found at R1 upstream of the studied WwTWs. This may be due to a high proportion of the small population using it upstream of this point and less dilution within the environment due to low river flows. Buprenorphine is commonly used for treatment of opioid addiction but can also be used for pain relief in patients who do not find relief with, or cannot use non-opioid analgesics (DrugBank [28]).

3.1.10.9 Drug precursors

Ephedrine was found in all environmental samples, but its metabolite was not. The parent compound was found at concentrations of 5.8 to 77.2 ng L^{-1}. This range is far below the PNEC, resulting in a $RQ_{MEC:PNEC}$ of 0.002. Norephedrine has a MQL of 8.82 ng L^{-1}, for this level the PNEC of 2,804 ng L^{-1} results in a $RQ_{MEC:PNEC}$ of 0.003. Overall, this class shows little risk to the environment.

3.1.10.10 Anticancer pharmaceuticals

Of the 7 anticancer pharmaceuticals, two were found in the environment. Imatinib was found in 12 out of 56 samples, at all sites apart from R6, at concentrations ranging from 6.9 to 144.3 ng L^{-1}. Bicalutamide was found in 55 out of 56 samples, at concentrations of 50.9 to 75.1 ng L^{-1}. Capecitabine was only found at low predicted concentrations at site R2 at 0.05 ng L^{-1}. PNEC could not be calculated for imatinib or capecitabine. The PNEC for bicalutamide was 1.0 µg L^{-1}, resulting in an $RQ_{MEC:PNEC}$ of 0.07. The most toxic anticancer agent, tamoxifen has a MQL of 72.6 ng L^{-1} this is far higher than the PNEC of 1.35 ng L^{-1}, resulting in and $RQ_{MEC:PNEC}$ of 53, therefore may pose a high risk to the aquatic environment at unquantifiable levels. The second compound with the second most toxic PNEC (16.80 ng L^{-1}) of this class, ifosfamide shows much lower risk of 0.02, due to it low MQL of 0.40 ng L^{-1}.

3.1.10.11 Anaesthetic and metabolite

Ketamine was found in all samples from R2 to R8, at concentrations of 50.9 to 75.1 ng L^{-1}. But the metabolite was not found. This is only slightly lower than the PNEC of 106 ng L^{-1}, resulting in $RQ_{MEC:PNEC}$ of 0.158 suggesting low to medium risk to the aquatic environment throughout the catchment.

3.1.10.12 Antidepressants

Of the 13 antidepressants and metabolites, two, venlafaxine and desmethylvenlafaxine were found in all samples throughout the catchment, mirtazapine was found in 49 samples, citaloprams metabolite, desmethylcitalopram, was found in 41 samples, amitriptyline in 35 and nortriptyline in 18. Citalopram and sertraline were found in 7 samples, only at R2. The highest concentration was seen for desmethylvenlafaxine, ranging from 45.6 to 258.1 ng L^{-1}, whilst the parent is present at 27.0 to 102.5 ng L^{-1}. The $RQ_{MEC:PNEC}$ for these compounds shows minimal risk for venlafaxine (RQ = 0.003), whereas desmethylvenlafaxine shows a higher risk to aquatic organisms with an $RQ_{MEC:PNEC}$ of 0.109. Citalopram and nortriptyline show higher levels of risk with $RQ_{MEC:PNEC}$ of 0.388 and 0.542, but amitriptyline (with environmental

concentrations of 2.2 to 15.5 ng L^{-1}), shows the highest environment risk with $RQ_{MEC:PNEC}$ of 0.908, close to the high risk level of 1. Overall, the mixture of antidepressants at the concentrations seen in the environment shows high risk to the environment with a $RQ_{mixture}$ of 1.546. The sum of toxic unit shows the highest risk is for algae, though the RQ_{STU} shows limited risk (0.003).

3.1.10.13 Antiepileptics

Carbamazepine and its metabolites were found in nearly all samples (10,11-dihydro-10-hydroxycarbamazepine was present in 55). Carbamazepine was present at concentrations of 60.3 to 185.1 ng L^{-1}, whereas carbamazepine-10,11-epoxide and 10,11-dihydro-10-hydroxycarbamazepine were present at concentrations of 11.1 to 36.3 ng L^{-1} and 1.3 to 30.8 ng L^{-1}. PNECs for the metabolites could not be calculated as ecotoxicity information from either literature or modelled data was not available. $RQ_{MEC:PNEC}$ was calculated as 0.06 for carbamazepine showing low risk to the environment.

3.1.10.14 Calcium Channel blockers

Verapamil was not found in the environment (MQL 0.02 ng L^{-1}). For diltiazem, measurable concentrations were only detected downstream of the WwTW with concentrations ranging from 1.88 to 10.41 ng L^{-1}. Verapamil and diltiazem show similar PNECs with 274 ng L^{-1} and 281 ng L^{-1} respectively. The $RQ_{MQL:PNEC}$ for verapamil shows little aquatic environmental risk. Diltiazem shows higher levels of risk but still very low $RQ_{MEC:PNEC}$ with 0.03. Overall, at these levels, this class shows little environmental risk.

3.1.10.15 Hypnotics

All hypnotics studies were found in the aquatic environment, temazepam and diazepam were found in 35 samples. Diazepam was found throughout the catchment at concentrations of 1.0 to 33.5 ng L^{-1} whereas temazepam was primarily found in the lower levels of the catchment at R5 to R8 at concentrations of 3.5 to 24.2 ng L^{-1}. Oxazepam was the most frequently found hypnotic (n = 42), but at the lowest oxazepam 0.01 to 4.8 ng L^{-1}. PNECs ranged from 402 ng L^{-1} (diazepam) to 895 ng L^{-1} (temazepam). All three individually posed little aquatic environmental risk with $RQ_{MEC:PNEC}$ of <0.03. The $RQ_{mixture}$ for the class reached 0.04 at R6, showing a combined low risk.

Table 3 Heat map for the acute environmental risk ($RQ_{MEC:PNEC}$) of each CEC to aquatic organisms at each river sampling site (R1 further point upstream, to R8 furthest point downstream). Gradient is from low risk (green) to high risk (red).

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
UV Filters	Benzophenone-1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Benzophenone-2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Benzophenone-3	0.033	0.047	0.032	0.028	0.044	0.055	0.033	0.024
	Benzophenone-4	0.008	0.014	0.010	0.024	0.009	0.015	0.025	0.026
Parabens	Methylparaben	0.001	0.001	0.001	0.001	0.004	0.001	0.001	0.001
	Ethylparaben	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Propylparaben	0.000	0.000	0.000	0.002	0.002	0.002	0.000	0.000
	Butylparaben	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Plasticizer	Bisphenol A	0.000	0.003	0.011	0.014	0.009	0.015	0.018	0.021
Steroid estrogens	E1	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000
	E2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	EE2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Antibiotics and Antibacterial	Sulfasalazine	0.007	0.008	0.013	0.018	0.007	0.011	0.007	0.008
	Clarithromycin	1.223	1.546	1.036	2.217	1.678	3.052	2.688	2.404
	Azithromycin	0.000	31.576	6.833	8.399	0.000	0.000	0.000	10.087
	Trimethoprim	0.000	0.011	0.006	0.010	0.007	0.017	0.014	0.012
	Sulfamethoxazole	0.001	0.002	0.001	0.002	0.001	0.002	0.002	0.002
	Triclosan	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Amoxicillin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Metronidazole	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Sulfadiazine	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Cefalexin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ofloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ciprofloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Tetracycline	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Danofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxytetracycline	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Chloramphenicol	0.000	0.111	0.000	0.000	0.000	0.000	0.000	0.192
	Penicillin G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Penicillin V	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Erythromycin	0.000	0.040	0.000	0.622	0.103	0.086	0.132	0.162
	Prulifloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Antifungal	Griseofulvin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ketoconazole	0.647	0.488	0.436	0.421	0.492	0.598	0.548	0.370
Hypertension	Valsartan	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001
	Irbesartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Lisinopril	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Candesartan Cilexetil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NSAIDs	Ketoprofen	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ibuprofen	0.000	0.000	0.001	0.002	0.002	0.003	0.002	0.003

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
	Naproxen	0.278	0.317	0.455	0.712	0.418	1.072	0.841	0.893
	Diclofenac	0.422	0.764	0.706	0.980	0.236	0.437	0.383	0.408
	Acetaminophen	0.006	0.000	0.004	0.016	0.017	0.022	0.022	0.022
Lipid regulator	Bezafibrate	0.007	0.011	0.014	0.021	0.014	0.022	0.019	0.020
	Atorvastatin	0.000	1.063	2.128	2.954	0.000	2.780	1.678	2.136
	Gemfibrozil	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.000
Antihistamine	Fexofenadine	0.005	0.008	0.006	0.008	0.004	0.008	0.014	0.018
	Cetirizine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GUD/ED	Sildenafil	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000
Antidiabetics	Metformin	0.000	0.014	0.204	0.252	0.166	0.246	0.285	0.276
	Gliclazide	0.178	0.238	0.262	0.385	0.065	0.106	0.238	0.266
	Sitagliptin	0.007	0.010	0.005	0.007	0.003	0.003	0.007	0.006
Cough suppressant	Pholcodine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Beta-blocker	Atenolol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Metoprolol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Propranolol	0.002	0.005	0.002	0.004	0.001	0.003	0.004	0.004
	Bisoprolol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H2 receptor agonist	Ranitidine	0.001	0.002	0.001	0.002	0.000	0.002	0.002	0.002
	Cimetidine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
X-ray contrast media	Iopromide	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Various	Buprenorphine	0.158	0.091	0.055	0.099	0.000	0.080	0.084	0.057
Drug precursor	Ephedrine/pseudoephedrine	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.001
	Norephedrine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Anticancer	Azathioprine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Methotrexate	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ifosfamide	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Tamoxifen	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Imatinib	0.063	0.003	0.014	0.017	0.014	0.000	0.024	0.018
	Capecitabine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Bicalutamide	0.006	0.007	0.005	0.006	0.007	0.006	0.008	0.007
Anaesthetic and metabolite	Ketamine	0.000	0.002	0.008	0.016	0.010	0.015	0.022	0.023
	Norketamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Venlafaxine	0.001	0.002	0.002	0.003	0.001	0.001	0.002	0.003
	Desmethylvenlafaxine	0.011	0.019	0.018	0.024	0.005	0.010	0.014	0.015
	Fluoxetine	0.000	0.237	0.000	0.045	0.000	0.000	0.000	0.000
	Norfluoxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sertraline	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000
	Mirtazapine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Citalopram	0.000	0.016	0.000	0.000	0.000	0.000	0.000	0.000
	Desmethylcitalopram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Paroxetine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Duloxetine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Amitriptyline	0.222	0.123	0.223	0.249	0.121	0.170	0.202	0.166

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
	Nortriptyline	0.000	0.027	0.026	0.059	0.016	0.008	0.060	0.061
	Norsertraline	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Antiepileptic	Carbamazepine	0.013	0.023	0.020	0.026	0.011	0.015	0.022	0.022
	Carbamazepine 10,11-epoxide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	10,11-Dihydro -10-hydroxycarbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Calcium-channel blocker	Diltiazem	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.000
	Verapamil	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Hypnotic	Temazepam	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Oxazepam	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Diazepam	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.002
Antipsychotic	Quetiapine	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000
	Risperidone	0.003	0.002	0.002	0.002	0.000	0.000	0.002	0.003
Dementia	Donepezil	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Memantine	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000
Lifestyle Chemicals and Creatinine	Creatinine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Nicotine	0.202	0.206	0.065	0.103	0.051	0.064	0.130	0.121
	Caffeine	0.003	0.002	0.003	0.004	0.003	0.005	0.006	0.006
	Cotinine	0.127	0.000	0.165	0.222	0.164	0.272	0.289	0.293
	1,7-dimethylxanthine	21.400	0.000	54.105	69.814	50.737	53.331	66.804	78.455
Opioid Analgesics and Metabolites	Heroin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	6-acetylmorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Morphine	0.000	0.019	0.000	0.000	0.000	0.017	0.021	0.021
	Dihydromorphine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Normorphine	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000
	Methadone	0.007	0.014	0.000	0.018	0.000	0.020	0.023	0.024
	EDDP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Codeine	0.025	0.045	0.061	0.105	0.052	0.110	0.102	0.110
	Norcodeine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Dihydrocodeine	0.000	0.001	0.001	0.002	0.002	0.003	0.002	0.002
	Tramadol	0.143	0.258	0.244	0.335	0.105	0.178	0.239	0.249
	N-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	O-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Stimulants and metabolites	Amphetamine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Methamphetamine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	MDMA	0.014	0.032	0.006	0.020	0.016	0.059	0.074	0.075
	MDA	0.033	0.038	0.026	0.028	0.000	0.000	0.000	0.000
	Cocaine	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.001
	Benzoylecgonine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Anhydroecgonine methylester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Cocaethylene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Mephedrone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDPV	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
Pesticides, fungicides and herbicides	Thiamethoxam	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Imidacloprid	0.000	0.001	0.002	0.005	0.000	0.000	0.000	0.000
	Clothiniadin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Metazachlor	0.000	0.000	0.474	0.204	0.000	0.000	0.000	0.000
	Terbuthylazine	0.000	0.000	0.000	0.000	0.107	0.000	0.000	0.000
	Methiocarb	0.000	0.000	0.475	0.000	0.000	2.548	0.543	2.173
	Dichlofluanid	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Flufenacet	0.004	0.004	0.003	0.003	0.004	0.004	0.004	0.004
	Oxadiazon	0.255	0.153	0.191	0.224	0.291	0.303	0.330	0.480
	Chlorpyrifos	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Triallate	0.285	0.000	0.000	0.000	0.000	0.000	0.000	0.423
Veterinary Pharmaceuticals	Tylosin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Sulfapyridine	0.025	0.039	0.018	0.024	0.011	0.014	0.013	0.016
	Sarafloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ceftiofur	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
	Diazinon	6.277	7.168	4.788	5.006	31.676	25.537	6.368	5.829

3.1.10.18 Antipsychotics

Quetiapine was only found at R6 in all samples, whereas risperidone was found in 16 samples across 7 of the sites. Concentrations were low and ranged from 0.51 to 0.9 ng L^{-1} for quetiapine and 0.01 to 3.96 ng L^{-1} for risperidone, these low levels cause minimal risk with $RQ_{MEC:PNEC}$ of 0.004 and 0.02 respectively.

3.1.10.19 Pharmaceuticals for treating dementia

Donepezil was not found in the environment (MQL 1.83 ng L^{-1}). Memantine was found in only 4 samples across the catchment and only at R2, therefore calculated from effluent rather than measured in the environment. Concentrations were calculated at 0.04 and 1.6 ng L^{-1}. Donepezil shows the higher level of risk as the PNEC is only ~10x higher than the MQL, $RQ_{MQL:PNEC}$ of 0.1, whereas memantine has a $RQ_{MEC:PNEC}$ of 0.01.

3.1.10.20 Lifestyle Chemicals and creatinine

Both caffeine (n = 53) and nicotine (n = 56) and their metabolites, 1,7-dimetylxanthine (n = 49) and cotinine (n = 49) were found throughout the environment, as concentrations ranging from 132.0 to 791.5 ng L^{-1}, 10.0 to 238.7 ng L^{-1}, 225.9 to 1,534.6 ng L^{-1}, and 16.5 to 67.5 ng L^{-1} respectively. Chronic ecotoxicity data was available for caffeine and nicotine, leading to PNEC of 3,100 µg L^{-1} and 2 µg L^{-1}, due to the availability of this data at three trophic levels an AF of 10 was applied. Combined with the MEC results in $RQ_{MEC:PNEC}$ of 0.0002 and 0.04 respectively. Similar data was not available for the metabolites, therefore chronic modelled data was replied upon for these. Due to this a high AF of 1000 was applied, combined with the high levels of the metabolite in environmental surface waters highlights this compound is of particular potential risk to the aquatic environment with $RQ_{MEC:PNEC}$ ranging from 7.7 to 283 from site R1 to R8. The sum of toxic units shows the algal trophic layer is most at risk with STU of 0.283. More robust ecotoxicity data is required to provide a more accurate ERA.

3.1.10.21 Opioid Analgesics and metabolites

Heroin and its metabolites were not found in the environment, with MQLs of 4.62 ng L^{-1} and 0.94 ng L^{-1}. Chronic ecotoxicity data was only available for heroin and for two trophic levels, therefore an AF of 100 was applied, this resulted in a PNEC of 63.9 ng L^{-1}. Considering the MQL, this indicates little to no risk of the unquantifiable concentrations, $RQ_{MQL:PNEC}$ of 0.07.

5 of the other 11 analgesics and metabolites were found in all samples in the environment (EDDP, codeine, tramadol, N-desmethyltramadol, and O-desmethyltramadol), dihydrocodeine was found in 49 samples, methadone in 42, and normorphine in 7. Dihydromorphine (MQL = 0.55 ng L^{-1}) and norcodeine (MQL = 9.52 ng L^{-1}) were not found. The PNEC for codeine and its metabolites is in the range of 1.1 to 1.8 µg L^{-1}. Tramadol has a higher PNEC with 3.5 µg L^{-1}, however data could not be obtained on the ecotoxicity of the metabolites. Morphine and its metabolites showed similar PNECs ranging from 0.14 to 0.19 µg L^{-1}. Methadone was shown to be the most toxic with a PNEC of 36.1 ng L^{-1}, but ecotoxicity data was could not be obtained for the metabolite. Overall, morphine poses the highest risk to the environment with $RQ_{MEC:PNEC}$ of 0.17, then methadone with $RQ_{MEC:PNEC}$ of 0.11 and tramadol with $RQ_{MEC:PNEC}$ of 0.09. The overall $RQ_{mixture}$ was 0.436 at the highest concentration of the catchment, for this class of compounds. The sum of toxic units show algae is most at risk, though this is still little to no risk at these levels.

3.1.10.22 Stimulants and metabolites

6 of the 10 stimulants and metabolites were not found in the environment (amphetamine, MQL 2.23 ng L^{-1}, methamphetamine, MQL 1.05 ng L^{-1}, anhydroecgonine methylester, MQL 4.67 ng L^{-1}, cocaethylene, MQL 0.35 ng L^{-1}, mephedrone, MQL 1.09 ng L^{-1}, MDPV, MQL 0.22 ng L^{-1}). The 4 found in the environment were MDMA (n = 54), cocaine (n = 56) and their metabolites, MDA (n = 28) and benzoylecgonine (n = 51) were found throughout at 0.1 to 31.8 ng L^{-1}, 0.3 to 13.5 ng L^{-1}, 7.7 to 14.8 ng L^{-1} and 14.2 to 106.5 ng L^{-1} respectively. Ecotoxicity data could not be obtained for anhydroecgonine methylester, cocaethylene, mephedrone and MDPV, therefore the risk for these CECS could not be determined. For amphetamine and methamphetamine the PNECs, 348 ng L^{-1} and 211 ng L^{-1} respectively, are far higher than the MQL, showing potentially unquantifiable levels of these compounds are of negligible risk to the aquatic environment. MDMA and its metabolite, MDA, present the highest risk, MDA in particular presents a medium risk to the aquatic environment with $RQ_{MEC:PNEC} > 0.1$ of 0.265. MDMA, cocaine and benzoylecgonine show low risk to the aquatic environment. Overall, the risk of this mixture of compounds at the concentrations found across the catchment show a combined risk of 0.299. Furthermore, unlike many classes and compounds these CEC pose the greatest risk at the top end of the catchment, where the flows cause less dilution of sources such as the WwTWs.

Table 4 Heat map for the chronic environmental risk ($RQ_{MEC:PNEC}$) of each CEC to aquatic organisms at each river sampling site (R1 further point upstream, to R8 furthest point downstream). Gradient is from low risk (green) to high risk (red).

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
UV Filters	Benzophenone-1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Benzophenone-2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Benzophenone-3	0.001	0.002	0.001	0.001	0.002	0.002	0.001	0.001
	Benzophenone-4	0.002	0.003	0.003	0.006	0.002	0.004	0.006	0.007
Parabens	Methylparaben	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ethylparaben	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Propylparaben	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Butylparaben	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Plasticizer	Bisphenol A	0.000	25.739	108.231	136.896	92.177	145.909	178.221	206.857
Steroid estrogens	E1	0.000	4.772	0.000	0.000	0.000	0.000	0.000	0.000
	E2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	EE2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Antibiotics and Antibacterial	Sulfasalazine	0.097	0.117	0.189	0.267	0.108	0.157	0.101	0.123
	Clarithromycin	0.152	0.192	0.129	0.276	0.209	0.379	0.334	0.299
	Azithromycin	0.000	0.270	0.058	0.072	0.000	0.000	0.000	0.086
	Trimethoprim	0.000	0.380	0.197	0.321	0.227	0.549	0.468	0.382
	Sulfamethoxazole	0.049	0.057	0.046	0.050	0.048	0.068	0.063	0.064
	Triclosan	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Amoxicillin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Metronidazole	0.000	0.006	0.013	0.011	0.009	0.021	0.016	0.014
	Sulfadiazine	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Cefalexin	0.001	0.000	0.000	0.000	0.001	0.001	0.005	0.002
	Ofloxacin	0.204	0.102	0.156	0.123	0.000	0.000	0.173	0.047
	Ciprofloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Tetracycline	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Danofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxytetracycline	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Chloramphenicol	0.000	0.204	0.000	0.000	0.000	0.000	0.000	0.355
	Penicillin G	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Penicillin V	0.002	0.000	0.002	0.000	0.001	0.000	0.000	0.001
	Erythromycin	0.000	0.183	0.000	2.875	0.476	0.399	0.610	0.750
	Prulifloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Antifungal	Griseofluvin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ketoconazole	3.515	2.651	2.368	2.287	2.677	3.254	2.976	2.012
Hypertension	Valsartan	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Irbesartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Lisinopril	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Candesartan Cilexetil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NSAIDs	Ketoprofen	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
	Ibuprofen	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Naproxen	0.000	0.000	0.000	0.001	0.000	0.001	0.001	0.001
	Diclofenac	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Acetaminophen	0.004	0.000	0.002	0.009	0.009	0.013	0.013	0.012
Lipid regulator	Bezafibrate	0.059	0.089	0.107	0.168	0.108	0.170	0.153	0.154
	Atorvastatin	0.000	1.054	2.110	2.929	0.000	2.756	1.664	2.118
	Gemfibrozil	0.000	0.002	0.000	0.000	0.000	0.031	0.000	0.000
Antihistamine	Fexofenadine	0.038	0.057	0.046	0.063	0.033	0.062	0.106	0.136
	Cetirizine	0.001	0.001	0.001	0.001	0.000	0.001	0.002	0.002
GUD/ED	Sildenafil	0.003	0.003	0.002	0.011	0.000	0.002	0.003	0.002
Antidiabetics	Metformin	0.000	0.004	0.066	0.082	0.054	0.080	0.093	0.090
	Gliclazide	1.073	1.433	1.583	2.321	0.390	0.641	1.436	1.606
	Sitagliptin	0.180	0.275	0.150	0.196	0.092	0.090	0.188	0.164
Cough suppressant	Pholcodine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Beta-blocker	Atenolol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Metoprolol	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000
	Propranolol	0.001	0.002	0.001	0.002	0.001	0.002	0.002	0.002
	Bisoprolol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H2 receptor agonist	Ranitidine	0.013	0.031	0.016	0.028	0.000	0.024	0.031	0.029
	Cimetidine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
X-ray contrast media	Iopromide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Various	Buprenorphine	1.004	0.579	0.349	0.626	0.000	0.507	0.532	0.363
Drug precursor	Ephedrine/pseudoephedrine	0.001	0.001	0.001	0.002	0.000	0.001	0.002	0.002
	Norephedrine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Anticancer	Azathioprine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Methotrexate	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ifosfamide	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Tamoxifen	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Imatinib	0.018	0.001	0.004	0.005	0.004	0.000	0.007	0.005
	Capecitabine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Bicalutamide	0.057	0.066	0.050	0.057	0.068	0.057	0.070	0.066
Anaesthetic and metabolite	Ketamine	0.000	0.013	0.057	0.111	0.071	0.099	0.151	0.158
	Norketamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Venlafaxine	0.002	0.003	0.002	0.003	0.001	0.002	0.003	0.003
	Desmethylvenlafaxine	0.049	0.086	0.079	0.109	0.024	0.043	0.065	0.066
	Fluoxetine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Norfluoxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sertraline	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000
	Mirtazapine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Citalopram	0.000	0.388	0.000	0.000	0.000	0.000	0.000	0.000
	Desmethylcitalopram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Paroxetine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
	Duloxetine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Amitriptyline	0.812	0.450	0.814	0.908	0.443	0.620	0.738	0.607
	Nortriptyline	0.000	0.240	0.233	0.525	0.142	0.075	0.539	0.542
	Norsertraline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antiepileptic	Carbamazepine	0.033	0.058	0.049	0.064	0.028	0.037	0.055	0.056
	Carbamazepine 10,11-epoxide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	10,11-Dihydro -10-hydroxycarbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Calcium-channel blocker	Diltiazem	0.000	0.017	0.000	0.016	0.000	0.025	0.000	0.000
	Verapamil	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Hypnotic	Temazepam	0.000	0.006	0.000	0.000	0.009	0.014	0.021	0.016
	Oxazepam	0.001	0.004	0.001	0.003	0.001	0.002	0.005	0.004
	Diazepam	0.017	0.029	0.016	0.016	0.016	0.015	0.016	0.016
Antipsychotic	Quetiapine	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000
	Risperidone	0.020	0.010	0.015	0.012	0.000	0.000	0.013	0.021
Dementia	Donepezil	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Memantine	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000
Lifestyle Chemicals and Creatinine	Creatinine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Nicotine	0.035	0.036	0.011	0.018	0.009	0.011	0.023	0.021
	Caffeine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Cotinine	0.004	0.000	0.005	0.006	0.005	0.008	0.008	0.008
	1,7-dimethylxanthine	77.215	0.000	195.219	251.899	183.068	192.426	241.039	283.079
Opioid Analgesics and Metabolites	Heroin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	6-acetylmorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Morphine	0.000	0.150	0.000	0.000	0.000	0.140	0.170	0.170
	Dihydromorphine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Normorphine	0.000	0.074	0.000	0.000	0.000	0.000	0.000	0.000
	Methadone	0.034	0.069	0.000	0.084	0.000	0.093	0.109	0.114
	EDDP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Codeine	0.014	0.026	0.035	0.060	0.030	0.063	0.059	0.063
	Norcodeine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Dihydrocodeine	0.000	0.015	0.009	0.020	0.022	0.039	0.018	0.020
	Tramadol	0.040	0.071	0.068	0.093	0.029	0.049	0.066	0.069
	N-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	O-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Stimulants and metabolites	Amphetamine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Methamphetamine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	MDMA	0.014	0.031	0.006	0.019	0.015	0.057	0.071	0.073
	MDA	0.233	0.265	0.184	0.199	0.000	0.000	0.000	0.000
	Cocaine	0.002	0.002	0.004	0.011	0.002	0.012	0.013	0.013
	Benzoylecgonine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Anhydroecgonine methylester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Cocaethylene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	R1	R2	R3	R4	R5	R6	R7	R8
	Mephedrone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDPV	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pesticides, fungicides and herbicides	Thiamethoxam	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Imidacloprid	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Clothiniadin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Metazachlor	0.000	0.000	2.102	0.905	0.000	0.000	0.000	0.000
	Terbuthylazine	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000
	Methiocarb	0.000	0.000	0.239	0.000	0.000	1.281	0.273	1.092
	Dichlofluanid	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Flufenacet	0.032	0.040	0.032	0.031	0.039	0.039	0.032	0.032
	Oxadiazon	0.722	0.434	0.542	0.636	0.824	0.857	0.935	1.360
	Chlorpyrifos	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Triallate	1.962	0.000	0.000	0.000	0.000	0.000	0.000	2.909
Veterinary Pharmaceuticals	Tylosin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Sulfapyridine	2.022	3.092	1.430	1.957	0.906	1.093	1.073	1.287
	Sarafloxacin	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Ceftiofur	0.000	0.008	0.031	0.038	0.000	0.000	0.000	0.000
	Diazinon	9.642	11.009	7.354	7.689	48.653	39.225	9.781	8.953

Table 5 Acute environmental risk ($RQ_{mixture}$) to aquatic organisms, for each class of CEC. Gradient is from low risk (green) to high risk (red).

Class	R1	R2	R3	R4	R5	R6	R7	R8
UV Filter	0.042	0.060	0.042	0.053	0.053	0.070	0.057	0.050
Parabens	0.001	0.001	0.001	0.002	0.006	0.003	0.001	0.001
Plasticizer	0.000	0.003	0.011	0.014	0.009	0.015	0.018	0.021
Steroid estrogens	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000
Antibiotics and antibacterial	1.234	33.294	7.890	11.268	1.797	3.168	2.844	12.868
Antifungal	0.647	0.488	0.436	0.421	0.492	0.598	0.548	0.370
Hypertension	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.001
NSAIDs	0.706	1.082	1.166	1.710	0.673	1.534	1.248	1.325
Lipid regulator	0.007	1.074	2.142	2.976	0.014	2.807	1.698	2.155
Antihistamine	0.005	0.008	0.006	0.008	0.004	0.008	0.014	0.018
GUD/ED	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000
Antidiabetics	0.184	0.261	0.472	0.644	0.234	0.356	0.530	0.549
Cough suppressant	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Beta-blockers	0.002	0.005	0.003	0.004	0.002	0.003	0.004	0.004
H2 receptor agonists	0.001	0.002	0.001	0.002	0.000	0.002	0.002	0.002
X-ray contrast media	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Various	0.158	0.091	0.055	0.099	0.000	0.080	0.084	0.057
Drug precursor	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.001
Anticancer	0.069	0.011	0.020	0.023	0.021	0.006	0.032	0.025
Anaesthetic and metabolite	0.000	0.002	0.008	0.016	0.010	0.015	0.022	0.023
Antidepressants	0.235	0.471	0.268	0.379	0.143	0.189	0.279	0.244
Antiepileptic	0.013	0.023	0.020	0.026	0.011	0.015	0.022	0.022
Calcium-channel blocker	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.000
Hypnotic	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.002
Antipsychotic	0.003	0.002	0.002	0.002	0.000	0.001	0.002	0.003
Dementia	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000
Human Indicators	21.732	0.209	54.337	70.143	50.955	53.671	67.229	78.875
Opioid Analgesics and Metabolites	0.175	0.347	0.306	0.459	0.159	0.328	0.386	0.405
Stimulants and metabolites	0.048	0.070	0.032	0.049	0.016	0.060	0.075	0.077
Pesticides, fungicides and herbicides	0.544	0.159	1.146	0.437	0.402	2.855	0.876	3.080
Veterinary Pharma	6.303	7.206	4.806	5.031	31.687	25.551	6.382	5.845

3.1.11.23 Pesticides, fungicides and herbicides

No CECs from this class was found in all environmental samples. Although many of these pesticides were prioritised due to their use in this catchment, data on their exact levels or frequency of use that year are unknown. Furthermore, the sampling was carried out during a dry period and surface runoff would have at a minimum. Chlorpyrifos, clothianidin, and thiamethoxam were not found in the environment (MQLs, of 42.9, 0.19, and 0.42 respectively). However, flufenacet was found in 55 samples. Concentrations in the environment between 1.2 ng L^{-1} (methiocarb) to 80.8 ng L^{-1} (imidacloprid). For the compounds that could not be found, $RQ_{MQL:PNEC}$, thiamethoxam and clothianidin the potentially unquantifiable concentrations show little to no risk to the environment. However, chlorpyrifos, has an exceptionally low PNEC of 0.05 ng L^{-1}, coupled with the MQL of 42.9 ng L^{-1}, the unquantifiable potential concentrations of this compound pose very high risk to the aquatic environment. For the CECs with quantifiable levels, 4 showed high risk to the environment, triallate, metazachlor, oxadiazon and methiocarb. At R1, R3 and R6, levels of triallate, metazachlor and methiocarb respectively were found at each site causing $RQ_{MEC:PNEC}$ to reach high risk to the environment. Three were found at R8 at levels which individually cause high risk to the environment. Overall, the $RQ_{mixture}$ at this site was 5.4. STU calculations showed the highest risk was to the daphnid trophic level, though with only RQ STU <0.01, showing low overall risk.

3.1.11.24 Veterinary pharmaceuticals

Of the veterinary pharmaceuticals, 3 were found in the environment. Ceftiofur was only found 3 times. Sulfapyridine and diazinon were found throughout, in 55 samples across the catchment. For these three compounds, concentrations ranged from 6.3 to 31.2 ng L^{-1}, 54.5 to 243.7 ng L^{-1} and 5.5 to 46.4 ng L^{-1} respectively. Sarafloxacin has an MQL of 6.39 ng L^{-1}, considering the PNEC the $RQ_{MQL:PNEC}$ show no risk to the environment at these levels. No publicly available ecotoxicity data could be found for tylosin and therefore the risk is unknown. For the remaining compounds ceftiofur shows little to no risk for the aquatic environment ($RQ_{MEC:PNEC}$ of 0.04), sulfapyridine shows high risk to the environment with RQ of 3.1, however, diazinon showed the highest risk to the aquatic environment with $RQ_{MEC:PNEC}$ of 48. Overall, this class shows a combined $RQ_{mixture}$ of 49.6. The STU shows highest risk to the daphnid trophic layer, with medium risk RQ_{STU} of 0.487. Highest risk was to site R5.

Table 6 Chronic environmental risk ($RQ_{mixture}$) to aquatic organisms, for each class of CEC. Gradient is from low risk (green) to high risk (red).

Class	R1	R2	R3	R4	R5	R6	R7	R8
UV Filter	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.01
Parabens	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Plasticizer	0.00	25.74	108.23	136.90	92.18	145.91	178.22	206.86
Steroid estrogens	0.00	4.77	0.00	0.00	0.00	0.00	0.00	0.00
Antibiotics and antibacterial	0.51	1.51	0.79	3.99	1.08	1.57	1.77	2.12
Antifungal	3.51	2.65	2.37	2.29	2.68	3.25	2.98	2.01
Hypertension	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NSAIDs	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01
Lipid regulator	0.06	1.14	2.22	3.10	0.11	2.96	1.82	2.27
Antihistamine	0.04	0.06	0.05	0.06	0.03	0.06	0.11	0.14
GUD/ED	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Antidiabetics	1.25	1.71	1.80	2.60	0.54	0.81	1.72	1.86
Cough suppressant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Beta-blockers	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
H2 receptor agonists	0.01	0.03	0.02	0.03	0.00	0.02	0.03	0.03
X-ray contrast media	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Various	1.00	0.58	0.35	0.63	0.00	0.51	0.53	0.36
Drug precursor	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Anticancer	0.08	0.07	0.05	0.06	0.07	0.06	0.08	0.07
Anaesthetic and metabolite	0.00	0.01	0.06	0.11	0.07	0.10	0.15	0.16
Antidepressants	0.86	1.17	1.13	1.55	0.61	0.74	1.34	1.22
Antiepileptic	0.03	0.06	0.05	0.06	0.03	0.04	0.05	0.06
Calcium-channel blocker	0.00	0.02	0.00	0.02	0.00	0.02	0.00	0.00
Hypnotic	0.02	0.04	0.02	0.02	0.03	0.03	0.04	0.04
Antipsychotic	0.02	0.01	0.01	0.01	0.00	0.00	0.01	0.02
Dementia	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Human Indicators	77.25	0.04	195.23	251.92	183.08	192.44	241.07	283.11
Opioid Analgesics and Metabolites	0.09	0.40	0.11	0.26	0.08	0.38	0.42	0.44
Stimulants and metabolites	0.25	0.30	0.19	0.23	0.02	0.07	0.08	0.09
Pesticides, fungicides and herbicides	2.72	0.47	2.91	1.57	0.88	2.18	1.24	5.39
Veterinary Pharma	11.66	14.11	8.81	9.68	49.56	40.32	10.85	10.24

3.1.11 Overall mixture assessment

Several CECs discussed throughout have shown RQ values greater than 1, therefore it is given that throughout the catchment $RQ_{mixture}$ values are also greater than 1, with $RQ_{mixtures}$ ranging from 99 to 517. This shows the mixture is of a considerable risk to the catchment, following commonly used mixture assessment guidelines [17, 34]. The acute and chronic RQ values can be found in Tables 7 – 10 for the aquatic environments. In particular, the high RQ at site R1 shows that other sources, such as much smaller WwTWs, septic tanks, surface runoff and other discharges, may still have high impact to the environment upstream in the catchment.

However, as stated by Backhaus et al, the use of $RQ_{mixture}$ calculated as per Equation 3 does not follow the standard rules of concentration addition in mixtures, as these RQs are based on the lowest toxicity for each individual CEC across three different trophic levels. For concentration addition the risk to each trophic level must be considered separately. This is done using Equation 4. The AF used for this mixture was 100, as the data included both modelled and literature-based data for three trophic levels. If the risk of the mixture was considered as a whole, the daphnid trophic layer is most at risk. In fact, the STU for this trophic level is greater than 1 for sites R3 (STU = 2.26) to R8 (STU = 4.25), without application of the assessment factor. Application of the AF show RQ_{STU}s greater than 10 from all sites. Furthermore, considering the contribution of the individual CECs to the total, BPA was identified as the primary source of toxicity at sites R3 to R8 ranging from 78% (R5) to 97% (R8). Diazinon contributes to most of the remaining ecotoxicity, ranging between 2.1% (R8) to 80% (R1). The high proportion at R1 is due to a lack of BPA upstream of the WwTWs. In a study focused only on pesticides by Ccanccapa et al. [104], diazinon has previously been found to be a similar level of high risk, where it was one of the most frequently found pesticides in the Ebro river basin, with a high chronic risk to aquatic invertebrates RQ of 24.2 in 2010 to 36.4 in 2011. Furthermore, in a study by Vallotton et al.[105], which investigated the hazard index and the maximum cumulative ratio (MCR), for 3099 mixtures. The study divided the mixtures into 4 groups, based on several implications; the mixture presents a risk, the mixture does not present a risk, the majority of the risk is based on one or two components, or the contribution of risk is more evenly distributed. In 20% of the mixtures where most of the risk is due to one or two components, diazinon is in the top 2. Considering these studies, the idea that one or two compounds of a mixture show the majority of the toxicological effect as suggested Backhaus and Karlsson, is supported [13]. However, this means that studies which do not cover a wide range of CECs may be underestimating the risk to the environment. For example, in a previous study by Gustavasson et al. [20], which investigated 172 CECs in Swedish coastal water found

triclosan, TBT and naproxen to be the top contributors to mixture RQs at different locations, however diazinon and BPA were not investigated.

This method contains 142 CECs from 32 classes, of the 138 of which were quantifiable in surface water, the average mixtures had between 57 (at R1) and 80 (at R2), with a median of 70 CECs at each site, and across the catchment the majority of the risk was due to diazinon and BPA. This is not an exhaustive list of CECs and there are many more which may pose a greater risk, as well as other priority substances that have been shown to be present in the environment and are not accounted for.

Furthermore, there are other factors to take into account when assessing a mixture with the concentration addition model; there is also very likely to be antagonistic and synergistic effects from different CECs. Future work would be required to explore the ecotoxicology of this mixture in the lab on three trophic levels, to better understand the full ramifications of this mixture when it is present in the environment.

Table 7 Acute aquatic RQs for the average mixture of CECs present at each of the sampled sites Gradient is from low risk (green) to high risk (red).

	R1	R2	R3	R4	R5	R6	R7	R8
$RQ_{mixture}$	32.11	44.88	73.17	93.77	86.69	91.34	82.36	106.03
RQ_{STU}	24.29	34.98	63.92	83.24	53.55	58.02	71.32	93.38

4

Table 8 Acute aquatic STU for the average mixture of CECs present at each of the sampled sites

	R1	R2	R3	R4	R5	R6	R7	R8
STU Daphnid	0.01	0.01	0.01	0.01	0.03	0.03	0.01	0.01
STU Fish	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
STU Algae	0.02	0.03	0.06	0.08	0.05	0.06	0.07	0.09
STU Max	0.02	0.03	0.06	0.08	0.05	0.06	0.07	0.09

Table 9 Chronic aquatic RQs for the average mixture of CECs present at each of the sampled sites

	R1	R2	R3	R4	R5	R6	R7	R8
$RQ_{mixture}$	99.38	54.92	324.42	415.12	331.06	391.51	442.56	516.50
RQ_{STU}	11.99	64.91	225.90	284.32	234.17	332.95	368.64	425.21

Table 10 Chronic aquatic STU for the average mixture of CECs present at each of the sampled sites

	R1	R2	R3	R4	R5	R6	R7	R8
STU Daphnid	0.12	0.65	2.26	2.84	2.34	3.33	3.69	4.25
STU Fish	0.01	0.10	0.01	0.01	0.00	0.01	0.01	0.01
STU Algae	0.10	0.03	0.22	0.29	0.21	0.24	0.28	0.32
STU Max	0.12	0.65	2.26	2.84	2.34	3.33	3.69	4.25

3.1.12 Spatial trends

With $RQ_{mixture}$ and RQ_{STU} ranging from 55 to 516 and 12 to 425 respectively (Table 9), there is high risk to the aquatic environment throughout the catchment. Sampling site R8, as the lowest point sampled downstream, is most at risk with $RQ_{mixture}$ of 517 and RQ_{STU} of 425 for the daphnid trophic layer (Table 10). This assessment does not consider the wider implication of damage to the daphnid trophic layer and the resultant effect on the ecological balance. Nor does it consider bioaccumulation or concentration within organisms, or the potential for these organisms to have adapted to this potentially pseudo-persistent mixture.

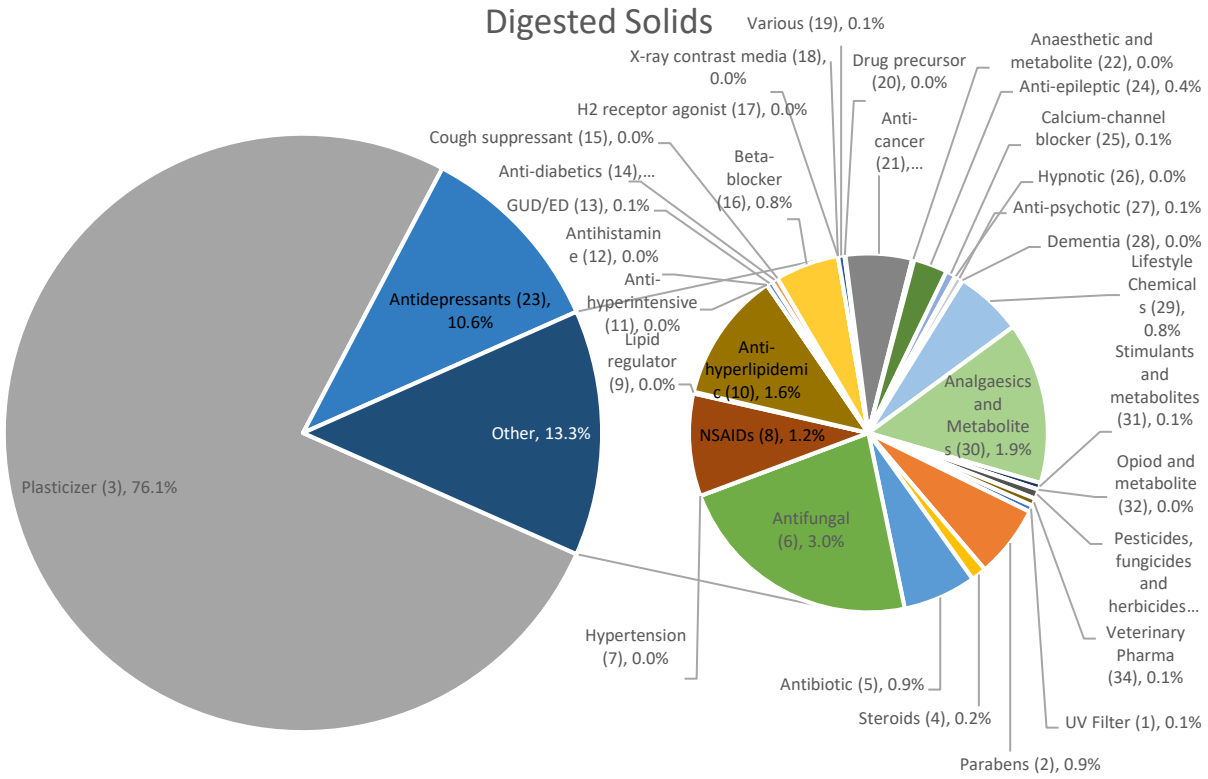
Although sampling at sites occurred in pairs (e.g. upstream and downstream), the entire catchment was not done in a single week. A clear, significant, increasing trend in average weekly concentrations can be seen throughout the catchment for certain CECs e.g BPA, clarithromycin, trimethoprim, ibuprofen, naproxen, acetaminophen, bezafibrate, metformin, caffeine, and oxadiazon (Table 1).

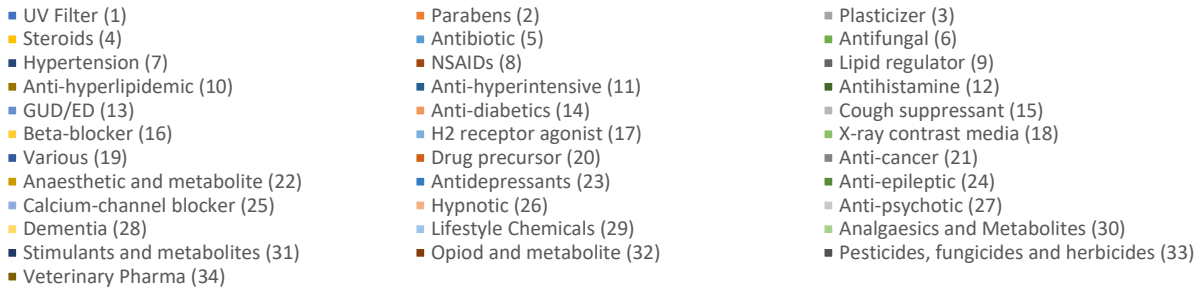
These compounds increase throughout the catchment and unlike many other compounds, do not significantly decrease between sites. Furthermore, many of these CECs, pharmaceuticals specifically, are those of which have been shown to have high steady usage throughout the catchment. The increasing levels from R1 to R8 potentially show a level of persistence and transport throughout the catchment, suggesting that the dilution and degradation within the environment of these CEC is unable to reduce these levels effectively, leading to increased risk to the environment.

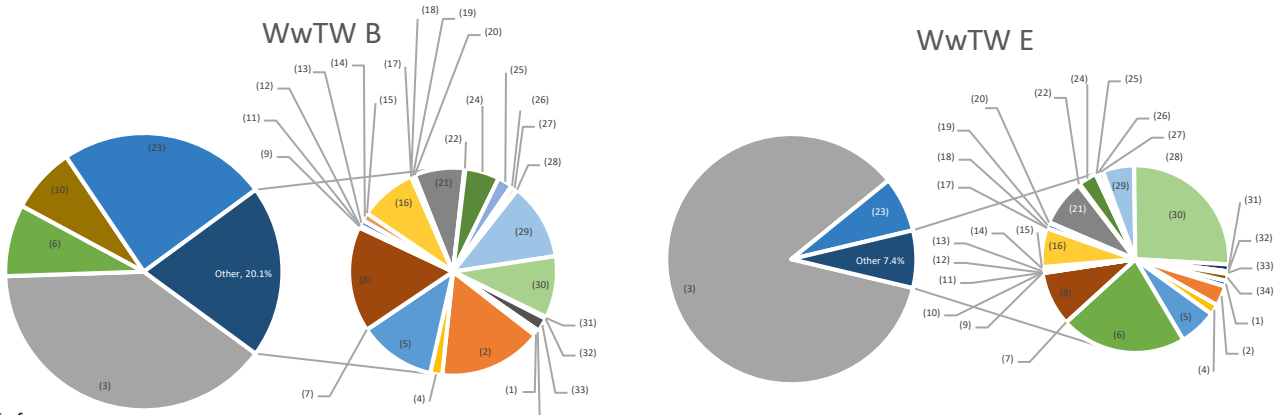
Overall, the plasticiser, BPA, lifestyle chemicals and veterinary pharma contribute the most risk to the catchment. The increasing levels of BPA can be attributed to its presence in everyday objects, such as receipts and plastics. It is leading to a significant increase in environmental risk through the catchment and can be attributed to the WwTW discharge, based on comparison of upstream and downstream sampling points.

However, there is a huge increase in chronic $RQ_{MEC:PNEC}$, >80, between downstream of WwTW A (R2) and upstream of WwTW B (R3), which cannot be attributed to any WwTW in this study and requires further investigation. For lifestyle chemicals, most of the risk is due to the caffeine metabolite, 1,7-dimethylxanthine, the main source of this can be attributed to WwTW discharges and potentially the degradation of caffeine in the environment; addressing the increases observed between sites. It also poses a significant risk to R1, with an $RQ_{MEC:PNEC}$ of 77.2, suggesting there must be a considerable source upstream.

For veterinary pharma, the main contributor to the overall toxicity of the class is diazinon, it was found at a reasonably consistent chronic level ,$RQ_{MEC:PNEC}$ between 7.4 (R3) and 11.0 (R2), however there is a significant increase to chronic $RQ_{MEC:PNEC}$ of 48.7 at R5, showing there is a potential source between WwTW B and C. However, although it was banned in 2009 for use as an insecticide in the EU, it may still be used illegally, resulting in its continued presence in the environment [106].







% of total	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
WwTW B	0.1	3.3	39.4	0.4	2.4	8.4	0.0	3.3	0.1	7.7	0.0	0.0	0.2	0.2	0.0	1.8	0.0	0.0	0.1	0.0	1.6	0.0	24.4	1.1	0.5	0.0	0.2	0.0	2.4	1.9	0.1	0.0	0.4	0.1
WwTW E	0.1	0.3	85.4	0.1	0.5	1.6	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.1	0.0	0.6	0.0	7.1	0.2	0.0	0.0	0.1	0.0	0.4	1.9	0.1	0.0	0.0	0.1

Figure 4 *Percentage of total concentration in digested solids of the entire catchment as a pie chart of classes, with individual pie charts for each site. Note: creatinine is not included. 0.0% shows negligible to no contribution to the total. Numbers in brackets indicate numbers assigned for identification in small figures and table (Taken from paper by Proctor et al.,* [21])

3.2. Occurrence in digested solids and environmental risk assessment

3.2.1. Terrestrial Environmental Concentrations

A route of entry of CECs to the terrestrial environment is via application of digested solids or treated wastewater to soils. In the UK, particularly in this catchment, application of digested solids is more common. In this study, WwTW B and E received and treated sludges from WwTWs within the catchment, and across three consecutive days, the digested solids were found to contain 65 CECs out of the 96 quantifiable with this method, across the two sites. Despite the long residence time of digested solids there was a high amount of variation for some compounds across the three days (Supplementary Information, Tables S6-8). The distribution of classes within this matrix can be seen in (Figure 4). The study by Proctor et al, [21] (Chapter 3) identified several classes in the digested sludge from this catchment that may be a particular risk to the environment; plasticisers, NSAIDs, antidepressants, antibiotics and antifungals, which is due to their high levels in digested sludge compared to other chemicals. Other notables CECs in the digested sludge are gemfibrozil, propranolol, carbamazepine, methylparaben, chloramphenicol, and nicotine.

Using Equation 2 described in Section 2.8.2, the PEC_{SOIL} was calculated for comparison with terrestrial ecotoxicity data. This information can be found in Table 11. The majority of compounds found were in the ng kg^{-1} to µg kg^{-1} range. Total CEC daily average concentrations ranged from 16.6 µg kg^{-1} at WwTW B to 64.5 µg kg^{-1} WwTW E.

The highest predicted concentration of a CEC in amended soils was BPA with 6.1 µg kg^{-1} from digested solids from WwTW B to 58.9 µg kg^{-1} from digested solids from WwTW E, with several other CECs with concentrations around 1 µg kg^{-1}, ketoconazole 0.92 µg kg^{-1} (WwTW E) to 1.8 µg kg^{-1} (WwTW B), gemfibrozil was only found at WwTW B and ranged from 0.94 µg kg^{-1} to 1.5 µg kg^{-1}, citalopram 1.1 µg kg^{-1} to 1.3 µg kg^{-1} (WwTW B), sertraline 0.75 µg kg^{-1} (WwTW B) to 1.1 µg kg^{-1} (WwTW E).

Table 11 PEC_{SOIL} (µg kg^{-1}) for digested solids from WwTW B, E and overall, calculated from measured digested solids data and Equation 2

Class of Analyte	Analyte	WwTW B Mean	WwTW B Max	WwTW B Min	WwTW E Mean	WwTW E Max	WwTW E Min	Overall Mean
UV Filter	Benzophenone-1	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-2	0.015	0.024	0.009	0.045	0.047	0.040	0.030
	Benzophenone-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-4	N/A	N/A	N/A	0.012	0.012	0.012	0.012
Parabens	Methylparaben	0.530	0.771	0.360	0.163	0.181	0.138	0.346
	Ethylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Propylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Butylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Plasticizer	Bisphenol A	6.42	6.96	6.11	54.4	58.9	45.7	30.4
Steroid estrogens	E1	0.061	0.064	0.058	0.085	0.090	0.078	0.073
	E2	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	EE2	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antibiotics and Antibacterial	Sulfasalazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Clarithromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Azithromycin	0.008	0.014	0.005	0.320	0.320	0.320	0.086
	Trimethoprim	0.016	0.022	0.012	0.012	0.014	0.011	0.014
	Sulfamethoxazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Triclosan	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metronidazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfadiazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cefalexin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ciprofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Danofloxacin	N/A	N/A	N/A	0.443	0.443	0.443	0.443
	Oxytetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Chloramphenicol	0.555	0.575	0.535	0.124	0.124	0.124	0.411
	Penicillin G	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Penicillin V	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Erythromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Prulifloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antifungal	Griseofluvin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ketoconazole	1.37	1.76	1.08	1.03	1.13	0.92	1.20
Hypertension	Valsartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Irbesartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Lisinopril	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Candesartan Cilexetil	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NSAIDs	Ketoprofen	0.341	0.415	0.301	0.248	0.267	0.222	0.294
	Ibuprofen	0.159	0.165	0.156	0.033	0.039	0.025	0.096
	Naproxen	0.039	0.045	0.035	0.136	0.142	0.130	0.087
	Diclofenac	N/A	N/A	N/A	0.079	0.079	0.079	0.079
	Acetaminophen	0.011	0.012	0.010	0.005	0.005	0.004	0.008
Lipid regulator	Bezafibrate	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Atorvastatin	1.25	1.46	0.94	N/A	N/A	N/A	1.25
	Gemfibrozil	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antihistamine	Fexofenadine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cetirizine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GUD/ED	Sildenafil	0.031	0.032	0.030	0.025	0.026	0.024	0.028
Diabetes	Metformin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Gliclazide	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sitagliptin	0.041	0.044	0.037	0.020	0.030	0.015	0.030
Cough suppressant	Pholcodine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Beta-blocker	Atenolol	N/A	N/A	N/A	0.036	0.039	0.035	0.036
	Metoprolol	N/A	N/A	N/A	0.004	0.004	0.004	0.004
	Propranolol	0.292	0.338	0.263	0.281	0.293	0.265	0.286
	Bisoprolol	N/A	N/A	N/A	N/A	N/A	N/A	N/A
H2 receptor agonist	Ranitidine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cimetidine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
X-ray contrast media	Iopromide	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Various	Buprenorphine	0.019	0.022	0.017	0.042	0.048	0.034	0.031
Drug precursor	Ephedrine/pseudoephedrine	N/A	N/A	N/A	0.008	0.011	0.007	0.008
	Norephedrine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Anti-cancer	Azathioprine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methotrexate	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class of Analyte	Analyte	WwTW B Mean	WwTW B Max	WwTW B Min	WwTW E Mean	WwTW E Max	WwTW E Min	Overall Mean
	Ifosfamide	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tamoxifen	N/A	N/A	N/A	0.041	0.041	0.041	0.041
	Imatinib	0.181	0.260	0.129	0.357	0.503	0.204	0.269
	Capecitabine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Bicalutamide	0.074	0.092	0.059	0.034	0.034	0.034	0.064
Anaesthetic and metabolite	Ketamine	0.004	0.005	0.004	0.013	0.014	0.013	0.008
	Norketamine	0.001	0.002	0.001	0.006	0.007	0.004	0.003
	Venlafaxine	0.186	0.231	0.160	0.094	0.097	0.090	0.140
	Desmethylvenlafaxine	0.033	0.036	0.030	0.025	0.028	0.022	0.029
	Fluoxetine	0.285	0.354	0.250	0.418	0.475	0.364	0.352
	Norfluoxetine	0.134	0.189	0.103	0.191	0.215	0.174	0.162
	Sertraline	0.831	0.978	0.750	1.07	1.09	1.06	0.952
	Mirtazapine	0.102	0.117	0.089	0.134	0.139	0.129	0.118
	Citalopram	1.15	1.31	1.07	1.23	1.27	1.19	1.19
	Desmethylcitalopram	0.435	0.564	0.358	0.538	0.551	0.525	0.487
	Paroxetine	0.003	0.009	0.000	0.007	0.008	0.005	0.005
	Duloxetine	0.026	0.037	0.018	0.030	0.035	0.024	0.028
	Amitriptyline	0.694	0.779	0.632	0.736	0.764	0.709	0.715
	Nortriptyline	0.093	0.124	0.075	0.105	0.106	0.103	0.098
	Norsertraline	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Anti-epileptic	Carbamazepine	0.175	0.198	0.161	0.150	0.160	0.143	0.162
	Carbamazepine 10,11-epoxide	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	10,11-Dihydro -10-hydroxycarbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Calcium-channel blocker	Diltiazem	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Verapamil	0.075	0.088	0.068	0.051	0.051	0.051	0.069
Hypnotic	Temazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diazepam	0.006	0.007	0.006	0.005	0.005	0.005	0.006
Anti-psychotic	Quetiapine	0.026	0.029	0.020	0.040	0.044	0.037	0.033
	Risperidone	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dementia	Donepezil	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Memantine	0.012	0.012	0.012	N/A	N/A	N/A	0.012
Creatinine	Creatinine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lifestyle Chemicals	Nicotine	0.334	0.418	0.281	0.205	0.224	0.178	0.269
	Caffeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cotinine	0.059	0.068	0.053	0.052	0.054	0.049	0.055
	1,7-dimethylxanthine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Analgaesics and Metabolites	Morphine	0.093	0.157	0.029	0.607	0.651	0.550	0.401
	Dihydromorphine	N/A	N/A	N/A	0.090	0.100	0.083	0.090
	Normorphine	N/A	N/A	N/A	0.040	0.040	0.039	0.040
	Methadone	0.019	0.023	0.017	0.058	0.058	0.057	0.038
	EDDP	0.056	0.102	0.031	0.280	0.293	0.266	0.168
	Codeine	0.066	0.154	0.019	0.052	0.058	0.049	0.059
	Norcodeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Dihydrocodeine	0.036	0.044	0.032	0.005	0.005	0.005	0.021
	Tramadol	0.051	0.065	0.041	0.070	0.072	0.068	0.061
	N-desmethyltramadol	0.025	0.036	0.019	0.032	0.035	0.029	0.028
	O-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Stimulants and metabolites	Amphetamine	N/A	N/A	N/A	0.026	0.026	0.026	0.026
	Methamphetamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDMA	0.009	0.011	0.008	0.037	0.038	0.036	0.023
	MDA	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cocaine	N/A	N/A	N/A	0.006	0.006	0.006	0.006
	Benzoylecgonine	N/A	N/A	N/A	0.005	0.006	0.004	0.005
	Anhydroecgoninemethylester	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cocaethylene	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Mephedrone	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDPV	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Opiod and metabolite	Heroin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6-acetylmorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pesticides, fungicides and herbicides	Thiamethoxam	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Imidacloprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Clothiniadin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metazachlor	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Terbuthylazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methiocarb	0.002	0.003	0.002	N/A	N/A	N/A	0.002
	Dichlofluanid	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class of Analyte	Analyte	WwTW B Mean	WwTW B Max	WwTW B Min	WwTW E Mean	WwTW E Max	WwTW E Min	Overall Mean
Veterinary Pharmaceuticals	Flufenacet	0.022	0.023	0.021	0.020	0.020	0.020	0.021
	Oxadiazon	N/A	N/A	N/A	0.043	0.043	0.043	0.043
	Chlorpyrifos	0.138	0.138	0.138	N/A	N/A	N/A	0.138
	Triallate	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tylosin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfapyridine	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sarafloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ceftiofur	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diazinon	0.022	0.028	0.017	0.055	0.087	0.031	0.042

3.2.2. Terrestrial ecotoxicity

Unlike the aquatic environment, where WwTWs continually discharge CEC containing effluent into the environment maintaining pseudo-persistent levels, the application of digested solids is a specific event(s) [18, 107]. Therefore, the highest risk to the terrestrial environment is shortly after this application. A review by Caracciolo et al shows that microbial communities, if not harmed by the application of CECs, can soon (days to weeks) reduce the initial levels for different pharmaceuticals [108].

There is a lack of ecotoxicity information with regards to the CECs in this study, acute ecotoxicity data could only be gathered for 21 out of the 65 quantifiable CECs (Table 12). Though there are many studies available on the effects of these CECs on a variety of different organisms or plants, they are mostly applicable to the aquatic environment, or multifactor tests [109], leading to lack of dose-response relationships for a single factor; the concentration of the CEC of interest. Data would ideally be required for three trophic levels as with aquatic toxicity. ECOSAR presents ecotoxicity data for organisms such as earthworms but no others (except for insects with regards to the neonicotinoid pesticides) [60]. Ideally, microorganisms such as bacteria, fungus or protozoa should be covered as well as invertebrates, insects and gastropods.

Though there is a focus on earthworm toxicity, the levels in this study suggest once mixed with soils the CECs, both individually and as a mixture, show little risk to the earthworms within the environment (RQs are predominantly far lower than 1 (Table 13)). However, more data is needed regarding the ecotoxicity to other trophic levels of terrestrial relevance. The digested solids themselves (Table 13), show high risk of acute toxicity to the earthworms in the environment for gemfibrozil in digested solids from WwTW B, with an acute $RQ_{MEC:PNEC}$ up to 7.0. Other CECs show a similar risk, such as diclofenac in digested solids from both sites shows medium risk with $RQ_{MEC:PNEC}$ up to 0.34 at WwTW B and high risk in digested solids from WwTW E with an $RQ_{MEC:PNEC}$ up to 1.1 and ketoconazole shows high risk at both sites with $RQ_{MEC:PNEC}$ 1.9 at WwTW E. Furthermore, BPA and methylparaben also show high risk at the chronic level with chronic $RQ_{MEC:PNEC}$ up to 1.3 for digested solids from WwTW B and $RQ_{MEC:PNEC}$ of 1.5 for digested solids from WwTW E (Table 14). To mitigate the risk of this matrix when applied to soils, it should be ensured that they are well mixed to avoid localised pockets of high concentration. Due to the lack of ecotoxicity data for most CECs, the $RQ_{mixture}$ could not be calculated for soil or digested solids.

Table 12 Acute terrestrial ecotoxicity data for soils amended with digested solids with references, assessment factor, notes and PNEC

Class of Analyte	Analyte	EC50 Invertebrate 96h mg kg^{-1}	Ref	EC50 Earthworm 14d mg kg^{-1}	Ref	EC50 Plant mg kg^{-1}	Ref	AF	PNEC mg kg^{-1}
UV Filter	Benzophenone-1	-		-		-			-
	Benzophenone-2	-		-		-			-
	Benzophenone-3	-		-		-			-
	Benzophenone-4	-		-		-			-
Parabens	Methylparaben	-		1989	[60]	56*	[110]		56
	Ethylparaben	-		1508	[60]	-		1000	1.51
	Propylparaben	-		1136	[60]	-		1000	1.14
	Butylparaben	-		850	[60]	-		1000	0.850
Plasticizer	Bisphenol A	-		-		-		1000	0.000
Steroid estrogens	E1	-		-		-			-
	E2	-		-		-			-
	EE2	-		-		-			-
Antibiotics and Antibacterial	Sulfasalazine	-		5951	[60]	-		1000	5.95
	Clarithromycin	-		4068	[60]	-		1000	4.07
	Azithromycin	-		3881	[60]	-		1000	3.88
	Trimethoprim	-		>2000	[111]	-		1000	2.00
	Sulfamethoxazole	-		1287	[60]	-		1000	1.29
	Triclosan	-		-		-		1000	0.000
	Amoxicillin	-		15509	[60]	-		1000	15.5
	Metronidazole	-		-		-			-
	Sulfadiazine	-		1720	[60]	-		1000	1.72
	Cefalexin	-		18176	[60]	-		1000	18.2
	Ofloxacin	-		-		-			-
	Ciprofloxacin	-		-		-			-
	Tetracycline	-		>2000	[111]	-		1000	2.00
	Danofloxacin	-		-		-			-
	Oxytetracycline	-		-		-			-
	Chloramphenicol	-		-		-			-
	Penicillin G	-		10289	[60]	-		1000	10.3
	Penicillin V	-		10699	[60]	-		1000	10.7
	Erythromycin	-		6709	[60]	-		1000	6.71
	Prulifloxacin	-		-		-			-
	Norfloxacin	-		-		-			-
Antifungal	Griseofluvin	-		-		-			-
	Ketoconazole	-		629	[60]	-		1000	0.629
Hypertension	Valsartan	-		6911	[60]	-		1000	6.91
	Irbesartan	-		-		-			-
	Lisinopril	-		34784	[60]	-		1000	34.8
	Candesartan Cilexetil	-		3482	[60]	-		1000	3.48
NSAIDs	Ketoprofen	-		64.8	[111]	293.7	[58]	100	0.648
	Ibuprofen	-		3077	[60]	-		1000	3.08
	Naproxen	-		90.5	[111]	-		1000	0.090
	Diclofenac	-		694	[111]	-		1000	0.694
	Acetaminophen	-		4597	[60]	-		1000	4.60
Lipid regulator	Bezafibrate	-		3275	[60]	-		1000	3.28
	Atorvastatin	-		141	[111]	-		1000	0.141
	Gemfibrozil	-		-		-			-
Antihistamine	Fexofenadine	-		-		-			-
	Cetirizine	-		-		-			-
GUD/ED	Sildenafil	-		1161	[60]	-		1000	1.16
Diabetes	Metformin	-		-		-			-
	Gliclazide	-		-		-			-
	Sitagliptin	-		1483	[60]	-		1000	1.48
Cough suppressant	Pholcodine	-		-		-			-
Beta-blocker	Atenolol	-		1633	[60]	-		1000	1.63
	Metoprolol	-		>2000	[111]	-		1000	2.00
	Propranolol	-		3299	[111]	-		1000	3.30
	Bisoprolol	-		-		-			-
H2 receptor agonist	Ranitidine	-		-		-			-
	Cimetidine	-		-		-			-
X-ray contrast media	Iopromide	-		-		-			-
Various	Buprenorphine	-		-		-			-
Drug precursor	Ephedrine/pseudoephedrine	-		-		-			-
	Norephedrine	-		-		-			-

Class of Analyte	Analyte	EC50 Invertebrate 96h mg kg^{-1}	Ref	EC50 Earthworm 14d mg kg^{-1}	Ref	EC50 Plant mg kg^{-1}	Ref	AF	PNEC mg kg^{-1}
Anti-cancer	Azathioprine	-		-		-			-
	Methotrexate	-		44137	[60]	-		1000	44.1
	Ifosfamide	-		7293	[60]	-		1000	7.29
	Tamoxifen	-		-		-			-
	Imatinib	-		-		-			-
	Capecitabine	-		-		-			-
	Bicalutamide	-		1123	[60]	-		1000	1.12
Anaesthetic and metabolite	Ketamine	-		-		-			-
	Norketamine	-		-		-			-
	Venlafaxine	-		-		-			-
	Desmethylvenlafaxine	-		-		-			-
	Fluoxetine	-		-		-			-
	Norfluoxetine	-		-		-			-
	Sertraline	-		-		-			-
	Mirtazapine	-		-		-			-
	Citalopram	-		-		-			-
	Desmethylcitalopram	-		-		-			-
	Paroxetine	-		-		-			-
	Duloxetine	-		-		-			-
	Amitriptyline	-		-		-			-
	Nortriptyline	-		-		-			-
	Norsertraline	-		-		-			-
Anti-epileptic	Carbamazepine	-		-		-			-
	Carbamazepine 10,11-epoxide	-		-		-			-
	10,11-Dihydro -10-hydroxycarbamazepine	-		-		-			-
Calcium-channel blocker	Diltiazem	-		2996	[60]	-		1000	3.00
	Verapamil	-		-		-			-
Hypnotic	Temazepam	-		828	[60]	-		1000	0.828
	Oxazepam	-		741	[60]	-		1000	0.741
	Diazepam	-		641	[60]	-		1000	0.641
Anti-psychotic	Quetiapine	-		-		-			-
	Risperidone	-		-		-			-
Dementia	Donepezil	-		-		-			-
	Memantine	-		-		-			-
Creatinine	Creatinine	-		1073	[60]			1000	1.07
Lifestyle Chemicals	Nicotine	-		-		-			-
	Caffeine	-		-		-			-
	Cotinine	-		944	[60]	-		1000	0.944
	1,7-dimethylxanthine	-		-		-			-
Analgaesics and Metabolites	Morphine	-		-		-			-
	Dihydromorphine	-		-		-			-
	Normorphine	-		-		-			-
	Methadone	-		-		-			-
	EDDP	-		-		-			-
	Codeine	-		-		-			-
	Norcodeine	-		-		-			-
	Dihydrocodeine	-		-		-			-
	Tramadol	-		-		-			-
	N-desmethyltramadol	-		-		-			-
	O-desmethyltramadol	-		-		-			-
Stimulants and metabolites	Amphetamine	-		-		-			-
	Methamphetamine	-		-		-			-
	MDMA	-		-		-			-
	MDA	-		444	[58]	128	[58]	1000	0.128
	Cocaine	-		3487	[60]	-		1000	3.49
	Benzoylecgonine	-		442523	[60]	-		1000	443
	Anhydroecgoninemethylester	-		-		-			-
	Cocaethylene	-		-		-			-
	Mephedrone	-		-		-			-
	MDPV	-		-		-			-
Opiod and metabolite	Heroin	-		5596	[60]	-		1000	5.60
	6-acetylmorphine	-		-		-			-

Class of Analyte	Analyte	EC50 Invertebrate 96h mg kg^{-1}	Ref	EC50 Earthworm 14d mg kg^{-1}	Ref	EC50 Plant mg kg^{-1}	Ref	AF	PNEC mg kg^{-1}
Pesticides, fungicides and herbicides	Thiamethoxam	6.48E-01	[60]	>1000	[58]	>0.4	[58]	1000	0.0004
	Imidacloprid	6.47E-01	[60]	-	[60]	-		1000	0.001
	Clothiniadin	6.02E-01	[60]	-	[60]	-		1000	0.001
	Metazachlor	-		-		-			-
	Terbuthylazine	-		-		-			-
	Methiocarb	-		-		-			-
	Dichlofluanid	-		488	[60]	-		1000	0.488
	Flufenacet	-		914	[60]	-		1000	0.914
	Oxadiazon	-		-		-			-
	Chlorpyrifos	-		-		-			-
	Triallate	-		257	[60]	-		1000	0.257
Veterinary Pharmaceuticals	Tylosin	-		-		-			-
	Sulfapyridine	-		1247	[60]	-		1000	1.25
	Sarafloxacin	-		-		-			-
	Ceftiofur	-		17816	[60]	-		1000	17.8
	Diazinon	-		-		-			-

*HC10 from Kim 2018, equivalent to PNEC

Table 13 Acute terrestrial $RQ_{PECsoil:PNEC}$ for amended soils and $RQ_{MEC:PNEC}$ for digested solids. Gradient is from low risk (green) to high risk (red).

		Acute $RQ_{PECsoil:PNEC}$					Acute $RQ_{MEC:PNEC}$				
Class	**Analyte**	**B**	**B Max**	**E**	**E Max**	**Average Both**	**B**	**B Max**	**E**	**E Max**	**Average Both**
UV Filters	Benzophenone-1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parabens	Methylparaben	9.46E-06	1.38E-05	2.91E-06	3.23E-06	6.19E-06	6.43E-03	9.37E-03	1.98E-03	2.20E-03	4.21E-03
	Ethylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Propylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Butylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Plasticizer	Bisphenol A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Steroid estrogens	E1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	E2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	EE2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antibiotics and Antibacterial	Sulfasalazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Clarithromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Azithromycin	2.15E-06	3.48E-06	8.25E-05	8.25E-05	2.22E-05	1.46E-03	2.37E-03	5.61E-02	5.61E-02	1.51E-02
	Trimethoprim	8.14E-06	1.09E-05	6.18E-06	6.98E-06	7.16E-06	5.54E-03	7.38E-03	4.20E-03	4.75E-03	4.87E-03
	Sulfamethoxazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Triclosan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metronidazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfadiazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cefalexin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ciprofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Danofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxytetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Chloramphenicol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Penicillin G	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Penicillin V	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Erythromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Prulifloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antifungal	Griseofluvin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ketoconazole	2.17E-03	2.80E-03	1.63E-03	1.79E-03	1.90E-03	1.48E+00	1.91E+00	1.11E+00	1.22E+00	1.29E+00
Hypertension	Valsartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	Acute $RQ_{PECsoil:PNEC}$					Acute $RQ_{MEC:PNEC}$				
		B	B Max	E	E Max	Average Both	B	B Max	E	E Max	Average Both
	Irbesartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Lisinopril	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Candesartan Cilexetil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NSAIDs	Ketoprofen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ibuprofen	5.26E-04	6.40E-04	3.82E-04	4.12E-04	4.54E-04	3.58E-01	4.35E-01	2.60E-01	2.80E-01	3.09E-01
	Naproxen	5.18E-05	5.36E-05	1.06E-05	1.26E-05	3.12E-05	3.52E-02	3.64E-02	7.22E-03	8.54E-03	2.12E-02
	Diclofenac	4.26E-04	4.95E-04	1.50E-03	1.57E-03	9.64E-04	2.90E-01	3.36E-01	1.02E+00	1.07E+00	6.56E-01
	Acetaminophen	N/A	N/A	1.13E-04	1.13E-04	1.13E-04	N/A	N/A	7.71E-02	7.71E-02	7.71E-02
Lipid regulator	Bezafibrate	2.39E-06	2.65E-06	9.88E-07	1.09E-06	1.69E-06	1.63E-03	1.80E-03	6.72E-04	7.42E-04	1.15E-03
	Atorvastatin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Gemfibrozil	8.87E-03	1.03E-02	N/A	N/A	8.87E-03	6.03E+00	7.03E+00	N/A	N/A	6.03E+00
Antihistamine	Fexofenadine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cetirizine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GUD/ED	Sildenafil	2.64E-05	2.78E-05	2.18E-05	2.27E-05	2.41E-05	1.79E-02	1.89E-02	1.48E-02	1.54E-02	1.64E-02
Antidiabetics	Metformin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Gliclazide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sitagliptin	2.74E-05	2.95E-05	1.36E-05	2.04E-05	2.05E-05	1.86E-02	2.00E-02	9.25E-03	1.39E-02	1.39E-02
Cough suppressant	Pholcodine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Beta-blocker	Atenolol	N/A	N/A	2.22E-05	2.39E-05	2.22E-05	N/A	N/A	1.51E-02	1.63E-02	1.51E-02
	Metoprolol	N/A	N/A	1.80E-06	1.80E-06	1.80E-06	N/A	N/A	1.23E-03	1.23E-03	1.23E-03
	Propranolol	8.85E-05	1.03E-04	8.52E-05	8.88E-05	8.68E-05	6.02E-02	6.97E-02	5.79E-02	6.04E-02	5.90E-02
	Bisoprolol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
H2 receptor agonist	Ranitidine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cimetidine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
X-ray contrast media	Iopromide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Various	Buprenorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Drug precursor	Ephedrine/pseudoephedrine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norephedrine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Anticancer	Azathioprine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methotrexate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ifosfamide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tamoxifen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Imatinib	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Capecitabine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Bicalutamide	6.55E-05	8.22E-05	3.07E-05	3.07E-05	5.68E-05	4.46E-02	5.59E-02	2.09E-02	2.09E-02	3.86E-02
	Ketamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	Acute $RQ_{PECsoil:PNEC}$					Acute $RQ_{MEC:PNEC}$				
		B	B Max	E	E Max	Average Both	B	B Max	E	E Max	Average Both
Anaesthetic and metabolite	Norketamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antidepressants	Venlafaxine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Desmethylvenlafaxine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Fluoxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfluoxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sertraline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Mirtazapine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Citalopram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Desmethylcitalopram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Paroxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Duloxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Amitriptyline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Nortriptyline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norsertraline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antiepileptic	Carbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Carbamazepine 10,11-epoxide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	10,11-Dihydro -10-hydroxycarbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Calcium-channel blocker	Diltiazem	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Verapamil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Hypnotic	Temazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diazepam	9.73E-06	1.02E-05	8.22E-06	8.50E-06	8.98E-06	6.62E-03	6.95E-03	5.59E-03	5.78E-03	6.10E-03
Antipsychotic	Quetiapine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Risperidone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dementia	Donepezil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Memantine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lifestyle Chemicals and Creatinine	Creatinine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Nicotine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Caffeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cotinine	6.25E-05	7.20E-05	5.49E-05	5.74E-05	5.87E-05	4.25E-02	4.90E-02	3.74E-02	3.90E-02	3.99E-02
	1,7-dimethylxanthine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Opioid Analgesics and Metabolites	Heroin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6-acetylmorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Morphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Dihydromorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	Acute $RQ_{PECsoil:PNEC}$					Acute $RQ_{MEC:PNEC}$				
		B	B Max	E	E Max	Average Both	B	B Max	E	E Max	Average Both
	Normorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methadone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	EDDP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Codeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norcodeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Dihydrocodeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	O-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Stimulants and metabolites	Amphetamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methamphetamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDMA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cocaine	N/A	N/A	1.76E-06	1.85E-06	1.76E-06	N/A	N/A	1.19E-03	1.26E-03	1.19E-03
	Benzoylecgonine	N/A	N/A	1.16E-08	1.40E-08	1.16E-08	N/A	N/A	7.91E-06	9.51E-06	7.91E-06
	Anhydroecgonine methylester	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cocaethylene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Mephedrone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDPV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pesticides, fungicides and herbicides	Thiamethoxam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Imidacloprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Clothiniadin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metazachlor	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Terbuthylazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methiocarb	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Dichlofluanid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Flufenacet	2.38E-05	2.48E-05	2.21E-05	2.21E-05	2.34E-05	1.62E-02	1.69E-02	1.50E-02	1.50E-02	1.59E-02
	Oxadiazon	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Chlorpyrifos	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Triallate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Veterinary Pharmaceuticals	Tylosin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfapyridine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sarafloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ceftiofur	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diazinon	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 14 Chronic terrestrial $RQ_{PECsoil:PNEC}$ for amended soils and $RQ_{MEC:PNEC}$ for digested solids. Gradient is from low risk (green) to high risk (red).

		Chronic $RQ_{PECsoil:PNEC}$					Chronic $RQ_{MEC:PNEC}$				
Class	**Analyte**	**B**	**B Max**	**E**	**E Max**	**Average Both**	**B**	**B Max**	**E**	**E Max**	**Average Both**
UV Filters	Benzophenone-1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzophenone-4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Parabens	Methylparaben	1.56E-03	2.27E-03	4.80E-04	5.32E-04	1.02E-03	1.06E+00	1.54E+00	3.26E-01	3.62E-01	6.93E-01
	Ethylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Propylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Butylparaben	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Plasticizer	Bisphenol A	1.74E-03	1.88E-03	1.47E-02	1.59E-02	8.23E-03	1.18E+00	1.28E+00	1.00E+01	10.83	5.59E+00
Steroid estrogens	E1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	E2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	EE2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antibiotics and Antibacterial	Sulfasalazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Clarithromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Azithromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfamethoxazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Triclosan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metronidazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfadiazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cefalexin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ciprofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Danofloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxytetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Chloramphenicol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Penicillin G	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Penicillin V	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Erythromycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Prulifloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antifungal	Griseofluvin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	Chronic $RQ_{PECsoil:PNEC}$					Chronic $RQ_{MEC:PNEC}$				
		B	B Max	E	E Max	Average Both	B	B Max	E	E Max	Average Both
	Ketoconazole	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Hypertension	Valsartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Irbesartan	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Lisinopril	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Candesartan Cilexetil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NSAIDs	Ketoprofen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ibuprofen	5.14E-04	6.26E-04	3.74E-04	4.03E-04	4.44E-04	3.50E-01	4.26E-01	2.54E-01	2.74E-01	3.02E-01
	Naproxen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diclofenac	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Acetaminophen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lipid regulator	Bezafibrate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Atorvastatin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Gemfibrozil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antihistamine	Fexofenadine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cetirizine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GUD/ED	Sildenafil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antidiabetics	Metformin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Gliclazide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sitagliptin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Cough suppressant	Pholcodine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Beta-blocker	Atenolol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metoprolol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Propranolol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Bisoprolol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
H2 receptor agonist	Ranitidine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cimetidine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
X-ray contrast media	Iopromide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Various	Buprenorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Drug precursor	Ephedrine/pseudoephedrine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norephedrine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Anticancer	Azathioprine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methotrexate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ifosfamide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tamoxifen	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Imatinib	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Capecitabine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	Chronic $RQ_{PECsoil:PNEC}$					Chronic $RQ_{MEC:PNEC}$				
		B	B Max	E	E Max	Average Both	B	B Max	E	E Max	Average Both
	Bicalutamide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Anaesthetic and metabolite	Ketamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norketamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antidepressants	Venlafaxine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Desmethylvenlafaxine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Fluoxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norfluoxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sertraline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Mirtazapine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Citalopram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Desmethylcitalopram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Paroxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Duloxetine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Amitriptyline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Nortriptyline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norsertraline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antiepileptic	Carbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Carbamazepine 10,11-epoxide	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	10,11-Dihydro -10-hydroxycarbamazepine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Calcium-channel blocker	Diltiazem	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Verapamil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Hypnotic	Temazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diazepam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Antipsychotic	Quetiapine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Risperidone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dementia	Donepezil	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Memantine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Lifestyle Chemicals and Creatinine	Creatinine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Nicotine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Caffeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cotinine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	1,7-dimethylxanthine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Opioid Analgesics and Metabolites	Heroin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6-acetylmorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Morphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Class	Analyte	Chronic $RQ_{PECsoil:PNEC}$					Chronic $RQ_{MEC:PNEC}$				
		B	B Max	E	E Max	Average Both	B	B Max	E	E Max	Average Both
	Dihydromorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Normorphine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methadone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	EDDP	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Codeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Norcodeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Dihydrocodeine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Tramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	N-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	O-desmethyltramadol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Stimulants and metabolites	Amphetamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methamphetamine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDMA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cocaine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Benzoylecgonine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Anhydroecgonine methylester	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Cocaethylene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Mephedrone	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	MDPV	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Pesticides, fungicides and herbicides	Thiamethoxam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Imidacloprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Clothiniadin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Metazachlor	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Terbuthylazine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Methiocarb	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Dichlofluanid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Flufenacet	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Oxadiazon	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Chlorpyrifos	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Triallate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Veterinary Pharmaceuticals	Tylosin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sulfapyridine	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Sarafloxacin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Ceftiofur	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	Diazinon	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

3.3. Anticipated and accidental exposure

Overall the MECs in the low ng L^{-1} to µg L^{-1} range (or ng kg^{-1} to µg kg^{-1} range), from the discharge of treated wastewater from WwTWs or application of digested sludge to soil, are anticipated levels of exposure.

In the aquatic environment, these levels are pseudo-persistent due to their continuous release. However, they may contain higher levels such as in the case of direct disposal of CECs to wastewater, which could be considered as accidental exposure. Several instances of direct or incidental disposal have been found within this catchment, such as the cases of fluoxetine, carbamazepine and ketoconazole as discussed by Proctor et al., and Petrie et al., [21, 112]. In these cases, higher levels are likely to pose much higher levels of risk to the environment, particularly where the majority of the pharmaceutical is usually metabolised and excreted in small amounts e.g. fluoxetine, where only 2.5 to 11% is excreted unchanged. Furthermore, the partitioning of the solid may also be affected, as in the case of carbamazepine [21], where direct disposal may have led to an unusually high relative load in one phase compared to the other.

Further consideration should be given towards the chirality of compounds, as the EF of directly disposed CECs may also be different and lead to increased environmental risk. For instance, fluoxetine is prescribed as a racemate, however, in humans the R-enantiomer is metabolised faster leading to an enrichment of the S-enantiomer in wastewater [113], therefore direct disposal would lead to higher levels of R enantiomer in the environment. Andrés-Costa et al., [114] carried out activated sludge simulating microcosms, which showed preferential microbial metabolism of S-enantiomer. In consideration of direct disposal this may lead to enrichment of R-enantiomer during activated sludge treatment at the WwTWs. In the same study this enantiomer was shown to be far more toxic (x27) than its antipode, to the protozoa, *Tetrahymena thermophila*, which is a microorganism found in activated sludge treatment and soil. Therefore, direct disposal of this CEC may result in an increase in the most toxic fluoxetine enantiomer in activated sludge, then digested sludge and then the environment, which is particularly hazardous to lower trophic levels. As discussed previously, inhibition of microbial communities can further affect the degradation of pharmaceuticals and may reduce treatment efficiency within the WwTW and microbial degradation with the environment.

CECs which are not removed efficiently though WwTW, or preferentially partition to solids, may pose far higher harm to the aquatic and terrestrial environments respectively, as the levels of direct disposal will not be mitigated by the treatment process. Furthermore, the low continual release of these CECs may alter the microbial communities leading to an enrichment of certain species, which

may degrade these contaminants more readily [115], reducing the persistence of CECs and their environmental impact. However sudden increases in CECs, especially pesticides and antibiotics, may alter the microbial communities significantly, reduce microbial degradation and increase the hazard to the environment.

4. Conclusion

The results calculated show the risk to the environment of the measured concentrations. There is a lack of ecotoxicity data for the majority of CECs in the aquatic and terrestrial environment, so risk is calculated, rather than true hazard, which is based on modelled data and assessment factors to fill the gaps in the data. Although the situation is improving for the aquatic environment, as availability of chronic ecotoxicity data has improved compared to 5 years ago [13]. Overall, this accurate measurement of exposure to organisms in the aquatic environment gives more confidence in the environmental risk assessment than previous PEC calculations.

Spatial and temporal variation of environmental exposure and risk is of critical importance. An environmental risk assessment (ERA) based on few samples or anomalous concentrations, poses the risk of a snapshot in time or in extreme circumstances and subsequently would not provide a representative ERA for the catchment [1]. However, understanding how much CECs concentrations fluctuates and what causes this, may be an important consideration for particularly sensitive organisms. In the case of pesticides or antibiotics, fluctuations may inhibit microbial communities and reduce biodegradation of CECs in the environment. Furthermore, this has shown that certain locations subjected to large community discharge will be associated with higher risk.

High acute risk was determined for 5 out of 138 CECs, and high chronic risk was determined for 10 out of 138 CECs. The mixture assessment for classes showed 6 classes had high acute risk, and 10 had high chronic risk. The overall mixture had total concentrations ranging from 2,604 ng L^{-1} to 13,817 ng L^{-1} in the river. Organisms in the daphnid trophic levels have been identified as the most sensitive organisms to this mixture, though the risk (RQ_{STU}) is low. However, the list of investigated CECs is not exhaustive, there are likely to be many more CECs and priority substances present in this environment, that are not accounted for in this study, and contributing to the overall ecotoxicological effects. Furthermore, there are other factors to take into account when considering the mixture as a whole, as concentration addition is a simple model. There may also be antagonistic and synergistic effects from different CECs. Further work is required on exploring the ecotoxicology of this mixture in the lab on three trophic levels to better understand its full environmental ramifications.

This work identifies several key areas that require further investigation:

1) Aquatic ERAs still require more chronic data covering three trophic levels for individual compounds.

2) Aquatic ERA requires environmental matrices to be analysed for as wide a range of CECs as possible, to ensure as many of the trace chemicals are covered in the subsequent analysis. It has been shown that a few minor compounds may dominate the overall toxicity of the mixture, this has been shown in this study across the catchment with BPA and diazinon [13]. Yet the 'critical' CECs may differ between studies, therefore necessitating a broad investigation to minimise the chance of overlooking the 'critical' CECs.

3) With regards to the terrestrial ERA, there is still a significant lack of data for this. With the PEC_{Soil}, in this study, in the ug kg^{-1} range, showing minimal risk overall but the data is so limited it is hard to be certain.

4) $RQ_{mixture}$ could not be determined with regards to the mixture and the terrestrial environment assessment, due to the lack of data, so could be reinvestigated when sufficient data becomes available.

5) The presence of many CECs in digested sludge with log K_{ow} lower than 3, suggesting these will have the highest mobility within the environment and may pose further risk to groundwater or surface water with leaching and runoff.

6) Very little information is present regarding the metabolites of these compounds. Furthermore, the significant increase in metabolite concentration in both WwTWs and the environment, show that EMA guidelines for metabolites may underestimate the risk these compounds pose to the environment, since metabolites that are excreted at <10% of the human body are deemed to pose no significant risk to the environment and are not investigated further [18]. The large increase in the concentration of these metabolites during wastewater treatment and potential increase during environmental microbial degradation may pose a much larger risk these guidelines suggest.

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Chapter 5

Development of novel approaches and further ecotoxicological considerations

1. Introduction

The work in previous chapters has identified the ubiquitous presence of CECs in the environment (Chapter 3). Following this, the potential risk of the mixtures present has been calculated for this catchment based on currently available ecotoxicity data (Chapter 4). Although this was possible for the aquatic environment, there was limited data available for the terrestrial environment which highlights a need for further, more readily available ecotoxicity data and suitable tests. Furthermore, there was a significant lack of chronic ecotoxicity data for most of the CECs analysed in both the aquatic and terrestrial matrices.

One aspect that is yet to be considered by this work is the stereochemistry of CECs in the environment and how this may affect their fate and their ecotoxicity. The stereochemistry of a pharmaceutical is known to be an important consideration for their medicinal effects (Evans and Kasprzyk-Hordern, 2014; Petrie et al., 2014), however this is still understudied for non-target organisms in the environment (Camacho-muñoz et al., 2019; Petrie et al., 2014; Ribeiro et al., 2012). Only a fraction of CECs that have been found in the environment by achiral methods have also undergone enantiomeric quantification. However, the studies that have been carried out have shown stereoselective partitioning between solid and aqueous phases (Sanganyado et al., 2017, 2016), stereoselective uptake in plants (Gao et al., 2016; Petrie et al., 2018; Sanganyado et al., 2017; Wang et al., 2013), enantioselective degradation (Fono and Sedlak, 2005; Sanganyado et al., 2017) and ecotoxicity to non-target organisms (Andrés et al., 2009; Petrie et al., 2014; Sanganyado et al., 2017; Stanley et al., 2007). This is further evidence that more stereochemical data is required for a wider range of compounds to begin to fully understand the impact of CECs and their enantiomers in the environment.

This chapter explores the currently unpublished work investigating the variation in stereochemistry for a select few CECs in the catchment-based study (Chapters 3 and 4). The work goes on to discuss the use of standardised toxicity testing to explore the toxicity of ephedrine, followed by fluoxetine, and further consideration of the importance of investigating metabolites and mixtures. Following this, development began on a new ecotoxicity test to improve upon the standardised test, with the aim of increasing the range of application. Both the standardised test and the new test will use the protozoa *Tetrahymena thermophila*, but the new test will aim to reduce the overall volume of the toxicant required. Protozoa are microorganisms that can be found in both aquatic and terrestrial environments,

as well as activated sludge. Furthermore, they have a very short lifespan; in 24 hours they go through 5-6 generations. Their small size and sensitivity to CECs make them ideally suited as an organism for ecotoxicity testing (Gerhardt et al., 2010; Gilron and Gransden, 1999). Therefore, studying the response of this organism to toxicant will provide crucial chronic ecotoxicity that is relevant for both the aquatic and terrestrial compartments.

2. Materials and methods

2.1. Materials and equipment

2.1.1. Chemicals

Chemicals used in this work include reference chemical potassium dichromate ($K_2Cr_2O_7$), sodium bicarbonate ($NaHCO_3$), calcium sulphate ($CaSO_4.2H_2O$), magnesium sulphate ($MgSO_4.7H_2O$), and potassium chloride (KCl), which were purchased at the highest purity bioreagent grade available from Sigma Aldrich (Gillingham, UK). 1R,2S-(-)-ephedrine ((-)-Eph), 1S,2R-(+)-ephedrine ((+)-Eph), 1S,2S-(+)-pseudoephedrine, ((-)-Pse), 1R,2R-(-)-pseudoephedrine ((+)-Pse), were of ≥98% purity and were purchased from Sigma Aldrich. Rac-Fluoxetine, rac-norfluoxetine and internal standard IS FL-d5 were purchased from LGC standards with >97% purity. Enantiomerically pure standards of fluoxetine and norfluoxetine were synthesised at the University of Bath, from purchased chiral starting materials R- and S- 3-chloro-1-phenyl-1-propanol, each with 99% enantiomeric excess. The synthesis is described in the following paper 'Enantioselective transformation of fluoxetine in water and its ecological relevance.'(Andrés-Costa et al., 2017). R-(-)-methamphetamine, S-(+)-methamphetamine, R-(-)-amphetamine, S-(+)-amphetamine, were purchased from Cerilliant, Sigma Aldrich at 1.0 mg mL^{-1} solutions in methanol. High purity deionised water was obtained from a MilliQ system (18.2 MΩ).

2.1.2. Standard toxicity test equipment

The standard toxicity test, Chronic Protoxkit F, was purchased from MicroBioTests (University of Ghent, Belgium). This is supplied with enough consumables, stock culture of *Tetrahymena thermophila*, food substrate and reconstitution medium for 6 tests. The change in optical density (OD) was detected using the Jenway Spectrophotometer 6305 with a 440 nm wavelength filter.

Further tests were carried out with independently sourced consumables, which were matched as closely as possible to those supplied with the test kit. Semi-macro (1.6 mL), macro (4.0 mL) polystyrene cuvettes with 10 mm light path, cuvette caps and screw cap Pyrex autoclavable culture tubes were purchased from Fisher Scientific. Terumo 1.0 mL insulin syringes with needle (27 g x 0.5") were purchased from Medisave (Weymouth, UK). *T. thermophila*, food substrate and reconstitution medium were purchased from Microbiotests.

2.1.3. **Development of nanocosm test**

24-well plates and 48-well plates were obtained from Greiner Bio One (Stonehouse, UK). The final well plates used were 48-well plates, from Thermo Fisher Scientific (Manchester, UK). Optical adhesive film plate cover was purchased from Applied Biosystems by Life Technologies, Thermo Fisher Scientific. Changes in OD were measured with the SpectraMax M3-R from Molecular Devices (Berkshire, UK) and purchased via VWR (Leicestershire, UK). SoftMax Pro software was used to program the plate reader and visualise the data. A TurboVap evaporator (Caliper, UK, 40˚C, N_2, 5 – 15 psi), was used to evaporate off methanol from the purchased 1.0 mg mL^{-1} solutions of enantiomerically pure amphetamine and methamphetamine standards.

2.1.4. **Analysis of enantiomeric fraction (EF) of CECs in environmental matrices**

The reference standards: R/S (±)-propranolol, R/S (±)-mirtazapine, R/S (±)-atenolol, R/S (±)-metoprolol, R/S (±)-fluoxetine, R/S (±)-citalopram, R/S (±)-venlafaxine, and 1R,2R/1S,2S (±)-tramadol were purchased from Sigma Aldrich and R/S (±)-desmethylcitalopram was purchased from Toronto Research Chemicals (TRC, Toronto, Canada). Internal standards R/S (±)-atenolol-d7, R/S (±)-mirtazapine-d3, R/S (±)-fluoxetine-d5, R/S (±)-citalopram-d6 was purchased from Sigma Aldrich and R/S (±)-propranolol-d7, R/S (±)-metoprolol-d7, were purchased from TRC. All solvents were HPLC grade and purchased from Sigma Aldrich. Formic acid and ammonium acetate were HPLC grade and obtained from Sigma Aldrich.

Consumables include: Whatman GF/F glass fibre filters with 0.7 µm pore size were purchased from Sigma Aldrich, Oasis MCX (mixed cation exchange, 60 mg) solid-phase extraction (SPE) cartridges, 0.2 µm PTFE filters (Whatman, Puradisc, 13 mm) and polypropylene liquid-chromatography (LC) vials were obtained from Waters (UK). All glassware used in sample preparation and analysis was deactivated with dimethyldichlorosilane (5% DMDCS in toluene, Sigma Aldrich).

Equipment used in the preparation and analysis of samples includes: ScanVac CoolSafe freeze dryer, (Lynge, Denmark), 800 W MARS 6 microwave and perfluoroalkoxy (PFA) microwave-assisted extraction (MAE) tubes (CEM, UK), Waters ACQUITY UPLC™ system (Waters, UK) with CBV column (chirobiotic V, 250 x 2.1 mm, I.D. 5 µm (Sigma Aldrich, UK, column packed with and antibiotic Vancomycin), guard column, 20 x 2.0 mm, I.D. 5 µm. Analysis was with Xevo TQD (Triple Quadrupole) Mass Spectrometer (Waters, UK) controlled by MassLynx 4.1 (Waters, UK) controls the

Waters ACQUITY system and the Xevo TQD. Data processing was carried out with TargetLynx software (Waters UK).

2.2. Methods

2.2.1. Preparation of synthetic freshwater

Synthetic freshwater was required for the standard microbiotest protocol (MicroBioTests, n.d.). This was prepared from purchased biological grade reagents. 96.0 mg of $NaHCO_3$, 120.0 mg $CaSO_4.2H_2O$, 123.0 mg $MgSO_4.7H_2O$ and 4.0 mg KCl were added to 1.0 L of high purity deionised water. This was sonicated for approximately 60 seconds to dissolve the powder. The solution was thoroughly mixed, and the pH was checked to be ~ 7.6. It was then stored in a sterile, airtight bottle in the dark at 4˚C, and has a shelf life of up to one month.

2.2.2. Standard microbiotest protocol

Each test cell contains protozoa, food, and a certain concentration of toxic substance in synthetic freshwater. The protozoa, food and overall volume remain consistent in all cells. The only variable is the concentration of the toxicant. At the start of the test (T0) the OD is measured with the spectrophotometer. Over the next 24 hours, as the population of protozoa grows, the food will decrease. The food causes increased levels of turbidity within the test cell. Therefore after 24 hours if the population is uninhibited or unaffected in its growth by the toxicant, the measurements of the OD should decrease. The chosen concentrations of the test should cover a range which inhibits the decrease in food by 80-100% of the control to 0-20% of the control, to allow a suitable range for the effect concentrations to be statistically calculated.

The standard microbiotest protocol (MicroBioTests, n.d.) was adhered to, although minor improvements to the protocol were made, which are highlighted and described where they occurred. The protocol consists of 5 steps and is as follows:

1) Preparation of toxicant dilution series

   There are two types of dilution series; range finding test and definitive test. The range finding test is for finding the appropriate range for the final definitive test.

   i. Range finding test

   Prepare a stock solution of the toxicant that should theoretically show 100% inhibition (C1). From this, prepare a 1:10 dilution series spanning 5 orders of magnitude, with 10 mL in each

vial. This is carried out by transferring 1.0 mL of C1 and 9.0 mL of synthetic freshwater to a test tube labelled C2. Mix thoroughly. Then transfer 1.0 mL of C2 and 9.0 of synthetic freshwater to a test tube labelled C3 and mixed this thoroughly. This process continue until there are five concentrations.

ii. Definitive test

From the results of the range finding test, 80-100% inhibition of population growth and 0-20% inhibition growth should be determined. If not, then the test should be repeated for a more appropriate range until these concentrations are found. The definitive test is prepared from the lowest concentration which causes 80 – 100% inhibition (this will be C1 in the definitive test) and the highest concentration which causes less than 20% inhibition (this will C5). The remaining concentrations (C2-C4) are prepared to cover the range between these two concentrations. If the range is one order of magnitude, the concentrations should be; C2 = 0.56 x C1, C3 = 0.32 x C1, C4 = 0.18 x C1, and C5 = 0.10 x C1. If the range is two orders of magnitude; C2 = 0.32 x C1, C3 = 0.1 x C1, C4 = 0.03 x C1 and C5 = 0.01 x C1. Use synthetic freshwater to dilute.

2) Preparation of ciliate inoculum

Carefully shake the stock *T. thermophila* culture vial to mix, then using a sterile syringe, extract 500 µL from the vial and transfer to semi-macro cuvette. Add 1.0 mL of deionised water. Cap the cuvette and shake gently to mix. Using the spectrophotometer at 440 nm wavelength, measure the OD of the vial. Calculate the amount of deionised water required to reach a theoretical OD value of 0.040, using equation 1 and 2.

$$F = ODvalue \div 0.040 \quad (1)$$

$$V = 0.5 \times (F - 1) \quad (2)$$

Where F equals the dilution factor, ODvalue is the initial OD measurement, and V is the dilution volume required to reach the theoretical OD measurement of 0.040. Transfer 500 µL of the inoculum in the semi-macro cuvette to the ciliate inoculum tube and add the dilution volume determined from equation 2. Cap the tube and mix thoroughly. This is important to regulate the ciliate population, as this will result in approximately 100 protozoan per mL in each of the final test cells (MicroBioTests, n.d.).

3) Preparation of the food substrate

Transfer one vial of reconstitution medium to the food substrate vial, close the vial and mix thoroughly.

4) Inoculation of the test cells

Label 12 macro cuvettes with C0 to C5 (each in duplicate). Add 2.0 mL of synthetic freshwater to the cuvettes labelled C0. For the remaining cuvettes, add 2.0 mL of each of the five concentrations to the corresponding labelled vials. Add 40 µL of food substrate and 40 µL of the prepared ciliate inoculum to each vial. Cap each cuvette.

5) OD measurements and incubation of test cells

Calibrate the spectrophotometer with 2.0 mL of distilled water in a macro cuvette. Then take each capped test cuvette, carefully invert several times to mix and measure the OD. Place the cuvettes in an incubator at 30˚C for 24 hours. Gently invert the cuvette to mix and repeat the OD measurement in the calibrated spectrophotometer. The OD measurement should decrease with population growth, as the OD measurement correlates with the quantity of food remaining in the cuvette. The more the population that grows in 24 hours, the more the food is eaten and therefore the lower the OD measurement. The test can be considered valid when the OD of the controls are reduced to 40% of the original OD measurement. If this does occur, place the cuvettes back in the incubator for another 2 hours before testing the OD again and repeat up to 3 times (until 30 hours) if necessary. If the tests do not pass the validation criteria by this point, they can be considered to have failed.

6) Data processing

The standard tox kit is supplied with software to calculate the % inhibition using equation 3 and the 24 h EC_{50} (also EC_{10}, EC_{20}, EC_{70} and EC_{90}).

$$\%\ inhibition_{(C1-C5)} = \left(\frac{\Delta OD_{(C1-C5)}}{\Delta OD_{C0}}\right) \times 100 \quad (3)$$

2.2.3. **Analysis of EFs in environmental matrices**

Suspended particulate matter (SPM) was filtered (using GF/F glass fibre filter paper with 0.7 µm pore size) from influent wastewaters and collected on seven consecutive days at each WwTW, as described in the catchment study by Proctor et al., (Proctor et al., 2020). Digested sludge was also collected on three consecutive days at the WwTWs with sludge treatment (WwTW B and E). The collected solids were frozen at -20 ˚C, freeze-dried, homogenised, weighed to 0.25 g (a note was taken of the mass to 5 decimal places), spiked with 50 µL of 1.0 mg mL^{-1} internal standard mix and extracted by MAE following the protocol described by Evans et al., (Evans et al., 2015).

Enantiomer separation and analysis was carried out with Waters ACQUITY UPLC™ system with CBV (Chirobiotic V) column coupled with Xevo TQD (Triple Quadrupole) mass spectrometer. The mobile phase used was 4 mM ammonium acetate in 100% methanol with 0.005% formic acid with a flow rate of 0.1 mL min^{-1} under isocratic conditions. The column temperature was 25 ˚C and injection volume was 20 µL. Nitrogen, supplied by a high purity nitrogen generator, was used as the nebulising and desolvation gas. Argon was used as the collision gas. Other mass spectrometer parameters include capillary voltage at 3.49 kV, source temperature at 150 ˚C, and desolvation gas flow was at 300 L h^{-1}. Data analysis was carried out using TargetLynx. The enantiomeric fraction (EF) was calculated using equation 4, where E1 is the concentration of (+)-enantiomer or the enantiomer with the lowest retention time and E2 is the concentration of (-)-enantiomer or the enantiomer with the highest retention time.

$$EF = \frac{E1}{E1+E2} \quad (4)$$

The EFs were calculated for atenolol, metoprolol, propranolol, venlafaxine, desmethylvanlafaxine, fluoxetine, mirtazapine, citalopram, desmethylcitalopram, amphetamine, methamphetamine, MDMA, MDA, and mephedrone.

3. Results and Discussion

3.1. Stereochemistry of CECs and environmental risk assessment

Since chiral analysis is completed with a different method than CEC quantification, the method has different minimum quantification limits (MQLs). Therefore, there are some instances where despite CECs being quantifiable in the same sample with the achiral method, the chiral methods could not quantify the compound and an EF could not be calculated. Despite many CECs being chiral and known to exhibit stereoselective biological activity in human medicine, but also some differences in

stereoselective ecotoxicity, there is a lack of information on their enantiomeric composition in wastewaters and the environment.

3.1.1. **Stereochemical variation within influent solid and liquid phases**

In the liquid phase (LP) of influent wastewater, the mean EF of studied CECs ranged from 0.21 for mirtazapine at WwTW D to 0.69 for fluoxetine at WwTW E (Table 1). These are within the range of previously reported EFs for chiral pharmaceuticals and illicit drugs in influent LP (Camacho-muñoz et al., 2019; Kasprzyk-Hordern and Baker, 2012; López-Serna et al., 2013; MacLeod et al., 2007; Sanganyado et al., 2016). There is limited data available in the literature on the enantiomeric composition of mephedrone in influent, however a previous paper by Castrignanò et al.

Table 1 Enantiomeric fractions of influent and effluent matrices throughout the catchment.

	Influent										Effluent									
	WwTW A		WwTW B		WwTW C		WwTW D		WwTW E		WwTW A		WwTW B		WwTW C		WwTW D		WwTW E	
Compound	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**
Atenolol	0.50 ±	0.01	0.50 ±	0.00	0.50 ±	0.00	0.50 ±	0.00	0.49 ±	0.00	0.58 ±	0.01	0.47 ±	0.00	0.49 ±	0.01	0.49 ±	0.02	0.55 ±	0.00
Metoprolol	0.46 ±	0.02	0.48 ±	0.05	0.45 ±	0.04	0.47 ±	0.02	0.42 ±	0.04	0.45 ±	0.01	0.42 ±	0.04	0.44 ±	0.03	0.38 ±	0.01	0.42 ±	0.02
Propranolol	0.44 ±	0.01	0.41 ±	0.01	0.42 ±	0.01	0.41 ±	0.02	0.40 ±	0.01	0.38 ±	0.01	0.36 ±	0.00	0.37 ±	0.01	0.39 ±	0.03	0.37 ±	0.01
Venlafaxine	0.39 ±	0.04	0.39 ±	0.04	0.44 ±	0.04	0.37 ±	0.04	0.33 ±	0.03	0.46 ±	0.07	0.45 ±	0.01	0.35 ±	0.06	0.44 ±	0.01	0.39 ±	0.01
Desmethylvenlafaxine	0.51 ±	0.03	0.50 ±	0.03	0.53 ±	0.02	0.49 ±	0.08	0.45 ±	0.02	0.49 ±	0.01	0.48 ±	0.01	0.49 ±	0.03	0.49 ±	0.01	0.46 ±	0.01
Fluoxetine	0.68 ±	0.02	0.68 ±	0.02	0.68 ±	0.02	0.67 ±	0.02	0.69 ±	0.02	0.65 ±	0.03	0.66 ±	0.01	0.68 ±	0.03	0.65 ±	0.02	0.63 ±	0.01
Mirtazapine	0.24 ±	0.03	0.27 ±	0.04	0.31 ±	0.02	0.21 ±	0.03	0.24 ±	0.05	0.29 ±	0.01	0.28 ±	0.02	0.27 ±	0.04	0.27 ±	0.01	0.27 ±	0.02
Citalopram	0.36 ±	0.02	0.37 ±	0.00	0.35 ±	0.01	0.36 ±	0.02	0.35 ±	0.01	0.31 ±	0.00	0.34 ±	0.01	0.36 ±	0.01	0.34 ±	0.02	0.32 ±	0.01
Desmethylcitalopram	0.43 ±	0.01	0.45 ±	0.01	0.43 ±	0.02	0.42 ±	0.01	0.42 ±	0.01	0.43 ±	0.02	0.42 ±	0.01	0.43 ±	0.01	0.41 ±	0.01	0.43 ±	0.01
Amphetamine	0.42 ±	0.02	0.42 ±	0.01	0.44 ±	0.01	0.40 ±	0.02	0.44 ±	0.02	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A
Methamphetamine	<MQL±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	N/A
MDMA	0.33 ±	0.04	0.34 ±	0.05	0.33 ±	0.05	0.32 ±	0.07	0.36 ±	0.05	0.17 ±	0.04	0.24 ±	0.05	0.28 ±	0.04	0.29 ±	0.03	0.19 ±	0.03
MDA	<MQL±	0.14	<MQL ±	0.25	0.53 ±	0.51	0.52 ±	N/A	0.56 ±	N/A	<MQL ±	0.02	<MQL ±	0.06	0.40 ±	0.08	0.20 ±	N/A	0.53 ±	N/A
Mephedrone	<MQL±	N/A	<MQL ±	0.03	<MQL ±	N/A	0.52 ±	N/A	<MQL ±	N/A	<MQL ±	N/A	<MQL ±	0.06	<MQL ±	N/A	0.48 ±	N/A	<MQL ±	N/A

(Castrignanò et al., 2016), reported EFs of mephedrone in influent LP ranging from 0.53 to 0.59. A later studied verified that the enrichment of mephedrone, in influent LP, with R-(+)-mephedrone, from the racemate form in which it is distributed, was due to human metabolism (Castrignanò et al., 2017).

In SPM, only pharmaceuticals and not the illicit drugs underwent chiral analysis. Overall, most pharmaceuticals saw no difference in EF between SPM and influent LP (Table 1 and 2). However, metoprolol showed a significant difference at WwTW C and WwTW D with 0.21 and 0.28 in SPM respectively, 0.45 and 0.47 in influent LP. Citalopram and its metabolite, desmethylcitalopram also showed a significant difference in EF between the two phases and this was more consistent across all WwTW, ranging from 0.60-0.62 in SPM across all sites and 0.35-0.37 in influent LP for citalopram and 0.57 to 0.59 in SPM to 0.42 to 0.45 in influent LP for desmethylcitalopram. This may be indicative of stereoselective partitioning for these compounds (Sanganyado et al., 2017).

Tramadol was not analysed with chiral methods in influent LP, but in SPM shows the widest range of EF from an average of 0.73 at WwTW A to an average of 0.20 at WwTW C. WwTW C and E (0.34) show the lowest EF, which may be linked to the presence of hospitals, however more investigation is needed for both phases.

With regards to temporal trends, there was little variance of EF for most CECs entering the WwTWs in either influent LP or SPM. However, mirtazapine, in SPM, showed a significant increase of EF at WwTW A, 0.61 on Friday compared to 0.30 – 0.43 for the rest of the week. This was not observed in influent. At the other WwTWs, the EF is relatively consistent between 0.26 and 0.30.

3.1.2. **Stereochemical variation within wastewater treatment works – aqueous removal**

MDMA showed a greater change in EF, at WwTWs with activated sludge treatment, a 49% and 47% decrease in EF at WwTW A and E. The WwTWs with trickling filters also showed a decrease in EF, which ranged from 28%, 17% and 9% at WwTW B, C and D respectively (Table 1). Overall MDMA showed an increase in EFs over the weekend (0.26, 0.35 and 0.28 at WwTW E, D and B, respectively) and lower over the week (0.14, 0.23 and 0.21 at WwTW E, D and B, respectively), but this was not seen at WwTW A or C. MDMA's metabolite, MDA, was largely undetected in effluent across the catchment. When it was detected, it showed the EF was highly variable.

Atenolol also showed variation in EF between WwTWs using activated sludge treatment or trickling filters. At WwTWs A and E the EF increased by 16% and 12% respectively, whereas WwTWs B, C and D decreased slightly, by 2-5%. The other beta-blockers showed an overall decrease of EF at all sites, except for metoprolol at WwTW E (1% increase), with change ranging from 3-19% decrease for

Table 2 Enantiomeric fractions of solid environmental matrices throughout the catchment.

	Digested Solids						Suspended particulate matter																	
	WwTW B			WwTW E			WwTW A			WwTW B			WwTW C			WwTW D			WwTW E					
Compound	**EF**		**SD**	**EF**		**SD**	**EF**		**SD**	**EF**		**SD**	**EF**		**SD**	**EF**		**SD**	**EF**		**SD**	**EF**		**SD**
Propranolol	0.45	±	0.01	0.45	±	0.02	0.44	±	0.03	0.43	±	0.01	0.45	±	0.02	0.44	±	0.02	0.43	±	0.02			
Mirtazapine	0.32	±	0.01	0.33	±	0.01	0.38	±	0.11	0.28	±	0.05	0.26	±	0.02	0.27	±	0.01	0.30	±	0.02			
Atenolol	0.47	±	0.06	-	±	-	-	±	-	-	±	-	-	±	-	-	±	-	-	±	-			
Metoprolol	0.41	±	0.01	0.36	±	0.06	0.50	±	0.02	0.48	±	0.05	0.21	±	0.07	0.28	±	0.03	0.37	±	0.07			
Fluoxetine	0.67	±	0.01	0.66	±	0.02	0.67	±	0.02	0.67	±	0.02	0.68	±	0.02	0.68	±	0.02	0.67	±	0.02			
Desmethylcitalopram	0.53	±	0.01	0.56	±	0.01	0.59	±	0.02	0.59	±	0.01	0.57	±	0.01	0.59	±	0.01	0.58	±	0.01			
Citalopram	0.61	±	0.00	0.61	±	0.02	0.62	±	0.01	0.61	±	0.01	0.60	±	0.01	0.62	±	0.01	0.62	±	0.01			
Venlafaxine	0.47	±	0.02	0.51	±	0.01	0.49	±	0.02	0.41	±	0.03	0.34	±	0.03	0.44	±	0.02	0.39	±	0.05			
Tramadol	0.16	±	0.01	0.30	±	0.05	0.73	±	0.08	0.49	±	0.07	0.20	±	0.05	0.57	±	0.12	0.34	±	0.16			

metoprolol, and 5-12% decrease for propranolol. The differences in EF between influent LP and effluent show stereoselective processes also occurred during wastewater treatment. The results for atenolol contradict what was previously found by Ribeiro et al. (Ribeiro et al., 2013), who found atenolol showed no enantioselectivity in activated sludge inoculum treatment. This is likely due to different microorganisms in the different activated sludge systems.

Temporal trends showed little variation of EFs in effluent across the week for the pharmaceuticals, with coefficients of variance less than 10%, apart from WwTW C (20%) and WwTW E (15%) for venlafaxine, and WwTW C for mirtazapine (13%). At WwTW C, the EF of venlafaxine was steady from Wednesday to Saturday with EFs in the range of 0.39 - 0.40. However, between Sunday and Tuesday, the EF drops to 0.28. Whereas at WwTW E, the EF shows an increasing trend across the week from 0.33 on Wednesday to 0.45 on the following Tuesday.

3.1.3. Stereochemical variation between effluent and river water

Venlafaxine showed a lower EF in effluent compared to river water upstream or downstream at WwTW C (0.35 to 0.48 and 0.50 respectively). However, desmethylvenlafaxine does not show significant differences between the three sampling points at WwTW C, or at any WwTW or river sampling site. Atenolol showed a significant increase between EF of upstream samples and downstream samples at WwTW A, from 0.47 to 0.57. This is likely due to the effluent discharge at this point, which had an EF of 0.58.

All other CECs analysed showed no significant difference between any samples, between effluent and river water upstream and downstream of the discharge point in which they were detected. This is similar to what was found in the same catchment by Camacho-Muñoz et al., (Camacho-Muñoz et al., 2019).

3.1.4. Stereochemical variation through a water course

Kasprzyk-Hordern et al. (Kasprzyk-Hordern and Baker, 2012), have previously shown that EFs of various CECs can change, not only within WwTWs, but also throughout a watercourse. Changes in EF in the aquatic environment were seen for MDMA, ephedrine/pseudoephedrine (diastereomeric fraction, DF), but venlafaxine and atenolol didn't show a significant change. For ephedrine, this shows an enrichment of (1R,2S)-ephedrine upstream and an enrichment of the least toxic (1S,2S)-pseudoephedrine downstream.

However, in this study few spatial trends were seen. Most CECs were consistent throughout the catchment when they were above the MQL (Table 3). MDMA was the only illicit drug that was detected throughout the catchment. At R1, it showed a relatively high EF (0.30) compared to further downstream.

Table 3 Enantiomeric fractions of river water matrices throughout the catchment.

	R1		R2		R3		R4		R5		R6		R7		R8	
Compound	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**	**EF**	**SD**
Atenolol	0.47	± 0.01	0.57	± 0.01	0.48	± 0.01	0.47	± 0.02	0.46	± 0.01	0.47	± 0.01	0.50	± 0.04	0.48	± 0.01
Metoprolol	<MQL	± N/A	0.46	± 0.02	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A
Propranolol	0.39	± 0.01	0.39	± 0.02	0.38	± 0.01	0.37	± 0.01	0.39	± 0.01	0.39	± 0.01	0.39	± 0.01	0.40	± 0.01
Venlafaxine	0.50	± 0.02	0.48	± 0.02	0.48	± 0.01	0.48	± 0.01	0.48	± 0.03	0.50	± 0.05	0.48	± 0.01	0.50	± 0.03
Desmethylvenlafaxine	0.49	± 0.01	0.50	± 0.01	0.44	± 0.01	0.47	± 0.01	0.50	± 0.02	0.48	± 0.01	0.48	± 0.01	0.48	± 0.01
Fluoxetine	<MQL	± N/A	0.64	± 0.02	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	0.64	± 0.12	0.68	± 0.03
Mirtazapine	<MQL	± N/A	0.30	± 0.01	0.28	± 0.02	0.27	± 0.02	<MQL	± N/A	0.27	± 0.02	0.29	± 0.02	0.27	± 0.04
Citalopram	0.31	± 0.01	0.31	± 0.01	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A
Desmethylcitalopram	0.40	± 0.02	0.42	± 0.02	0.40	± 0.01	0.41	± 0.01	0.40	± 0.05	0.42	± 0.02	0.42	± 0.02	0.42	± 0.02
Amphetamine	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A
Methamphetamine	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A
MDMA	0.30	± 0.04	0.17	± 0.03	0.19	± 0.05	0.22	± 0.04	0.28	± 0.04	0.25	± 0.04	0.25	± 0.05	0.25	± 0.05
MDA	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A
Mephedrone	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A	<MQL	± N/A

At R2 the EF was 0.17, which was likely due to the discharge from the WwTWs which also had an EF of 0.17. From this point on however, the EF slowly rose throughout the catchment to 0.28 at R5 and then remained at 0.25 at the remaining sampling points.
Atenolol also showed an EF change related to discharge between river sampling points R1 (EF = 0.47) and R2 (0.57), again this is likely due to the discharge of WwTW A, which had a very similar EF (0.58).

Overall, the similarities of EF between the effluent sampling point and downstream sampling point may indicate the proximity of R2 is too close to WwTW A, as previously discussed in chapters 3 and 4. The results for venlafaxine contrasts to what was previously observed by Li et al. (Li et al., 2013), would found venlafaxine consistently decreased via stereoselective microbial degradation.

3.1.5. **Digested solids**

Enantioselective analysis of sludge extracts revealed chiral CECs were not present in the racemic form. Average EFs ranged from 0.16 for tramadol to 0.67 for fluoxetine (Table 2) and are similar to the limited work done on this area previously (Evans et al., 2015). For most CECs, no significant differences in EF were observed between sludge produced by WwTWs B and E. Overall, Figure 1, shows similar EFs present in digested sludge as in SPM, with the exception of tramadol, which shows high variance in SPM as well.

Beta-blockers have previously shown stereoselective biodegradation during wastewater treatment (Camacho-Muñoz et al., 2016; Fono and Sedlak, 2005; López-Serna et al., 2013; MacLeod et al., 2007; Vazquez-Roig et al., 2014), whereas this comparison between EFs in SPM and digested solids suggests this is not a factor for the treatment of solids, likely due to more physical thermal degradation rather than microbial.

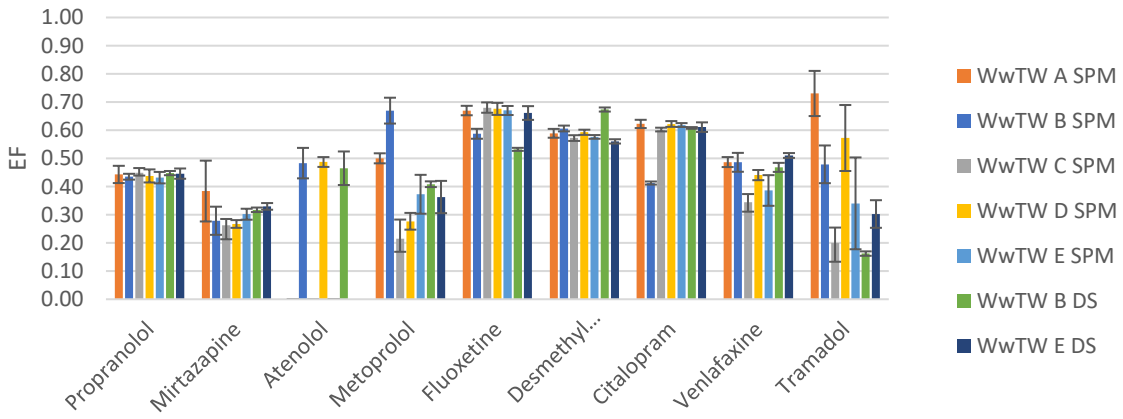


Figure 1 Average EF of seven consecutive days for SPM and three consecutive days for digested solids (DS) at different sites. Error bars show standard deviation.

3.1.6. **Preliminary conclusions**

This work so far has indicated that sorption to SPM maybe stereoselective, for metoprolol, citalopram and desmethylcitalopram. Whilst this observation requires further confirmation, it may be of critical importance if this trend can also be seen in the aquatic and benthic environment.

Removal from the liquid phase was also stereoselective for many compounds, with significant changes in EF between the two matrices. Differences possibly resulting from the two treatment types could also be seen for atenolol and MDMA.

In this catchment, the patterns of EF between effluent discharge, river upstream and downstream are very consistent, and indicate that the stereochemistry within the environment is highly influenced by the effluent. Furthermore, there is comparatively little stereochemical variation in river water for CECs throughout this catchment compared to previous studies, which indicates a lack of stereoselective microbial degradation. This was also seen for most CECs in the study by Camacho-Muñoz et al., (Camacho-Muñoz et al., 2019), however that work was further supported by lab-based river water microcosms. This practice should be a further consideration for this work.

Regarding the changes of EF in solids, further work is still required as they, prior to treatment, are a mixture of solids from the WwTW and tankered sludge from other WwTWs. Therefore, the contribution of SPM from each source to this mixture is unknown and further investigation would be required to fully understand the process.

Overall, few of these chiral CECs are racemic (50:50 ratio) in the environment (Table 1). This is of critical importance, as shown by the two papers following this work (Andrés-Costa et al., 2017; Rice et al., 2018), as different enantiomers can have different levels of effect and one may be far more toxic than another. Currently this is not taken into account in ERAs unless the pharmaceutical is sold as a pure enantiomer or human metabolism produces >10% of the other enantiomer. Therefore, changes within this ratio from wastewater treatment processes and within the environment are not considered. The ERAs of chiral CECs need to be treated as a mixture of enantiomers, or different compounds, to provide a more accurate risk assessment. Furthermore, as evidenced by the papers by Andrés-Costa et al. (Andrés-Costa et al., 2017),or Sanchez et al. (Sánchez et al., 2004) these enantiomers or their metabolites may have a synergistic or antagonistic effect on each other. This is discussed in more detail in the next section.

3.2. Application of the *T. thermophila* standard test protocol and further consideration towards future ecotoxicity tests and ERA

3.2.1. Application of the T. thermophila standard test protocol

The standard test protocol was used, as part of a battery of toxicity tests of various trophic levels, to explore the environmental significance of the varying stereochemistry found within wastewater treatment works and the environment, of ephedrine and fluoxetine. The context of this collaborative work and the results can be found in the following two published pieces (Andrés-Costa et al., 2017; Rice et al., 2018). The main conclusions for the studies are as follows:

3.2.1.1. Organism sensitivity towards differences in stereochemistry

Both studies (Andrés-Costa et al., 2017; Rice et al., 2018) show the importance of understanding enantioselective transformation within the WwTWs and the environment, and how this may differ from the human metabolism. This consideration is a key factor that is not taken into account with current guidelines for the ERA for new medicinal products (Camacho-Muñoz et al., 2016; European Medicines Agency, 2018). Furthermore, these pieces show the importance of considering the effects across different trophic levels. Not only to illustrate which trophic level is most at risk in the environment but to understand how some organisms may be more affected by differences in stereochemistry than others. For example, fluoxetine is far more toxic to the fish *P. promelas* than *D. magna* or *T. thermophila* (Andrés-Costa et al., 2017; Stanley et al., 2007). For the endpoint with the lower LOEC for this organism, (S)-fluoxetine is 3.4 times more effective than the (R)-enantiomer. Whereas, *D. magna*, a model organism and frequently used in ecotoxicity tests, shows a negligible difference between the two enantiomers. However, for *T. thermophila* the difference is clear, showing the (R)-enantiomer is 27 times more toxic than the (S)-enantiomer. Furthermore, this sensitivity to stereochemistry has been shown to vary between the diastereomers of ephedrine and fluoxetine for the lower trophic levels, as despite showing negligible difference between the enantiomers of fluoxetine, *D. magna* shows greater relative difference in toxicity between the two enantiomers of pseudoephedrine. *T. thermophila* on the other hand, shows a small difference in toxicity between the (1S,2R) and (1R,2S)-enantiomers of ephedrine (42.6 mg L^{-1} compared to 36.0 mg L^{-1}). Yet the enantiomers, (1S,2S)- and (1R,2R) of pseudoephedrine, differ by almost 22 times.

Some of this difference may be due to the ability of these organisms to adapt to what is entering the environment consistently at a low level. For example, of the four diastereomers of ephedrine, the two that have natural sources such as ‘ephedra’ ((1S,2S)-pseudoephedrine and (1R,2S)-ephedrine) are both

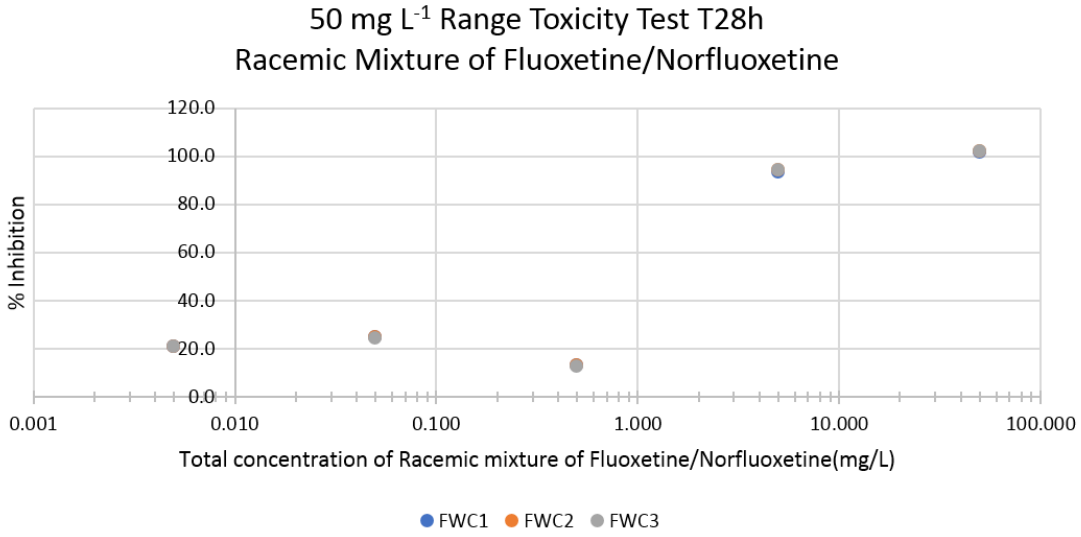
frequently used in medicine, are the most abundant in the environment (Kasprzyk-Hordern and Baker, 2012) and are also the least toxic enantiomers for *D. magna,* and (1S,2S)-pseudoephedrine is the least toxic enantiomer to all three trophic levels (Rice et al., 2018).

3.2.1.2. *Stereochemistry of ephedrine highlights the potential issue of seasonal potency*

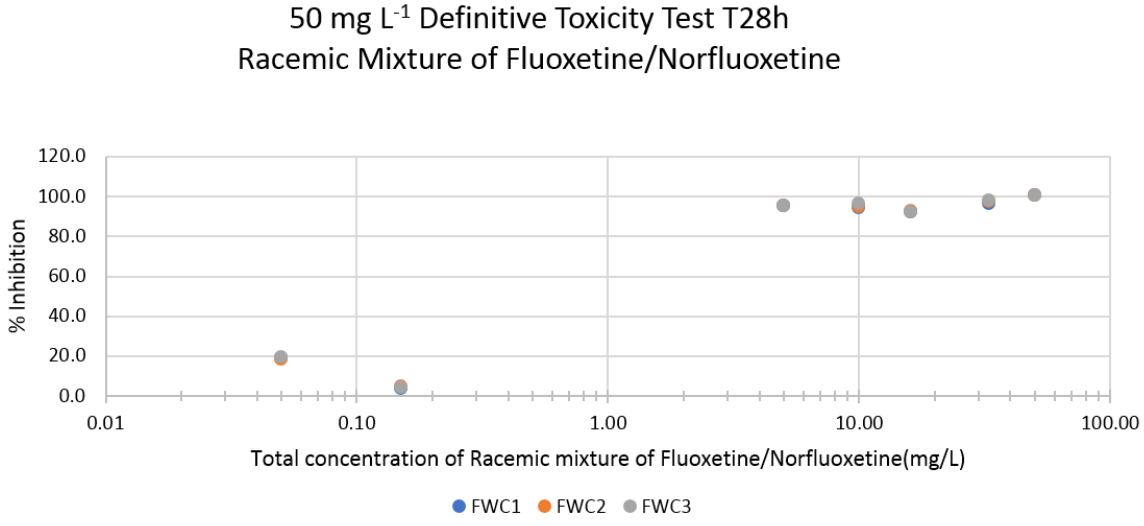
The ephedrine paper, (Rice et al., 2018), also highlights the potential issues with variation in seasonal potency of an environmental mixture with regards to the diastereomers of ephedrine, with the cumulative loads of these compounds reaching 180 g d^{-1} in winter (February) compared to <80 g d^{-1} in summer (August) (Kasprzyk-Hordern and Baker, 2012). At first glance, the winter loads might be considered a greater environmental risk than those seen in summer, however, the winter loads were enriched with the least toxic diastereomers (1S,2S)-pseudoephedrine and the summer ones were enriched with the more toxic (1R,2S)-ephedrine (Rice et al., 2018).

3.2.2. **Mixtures**

The standard test protocol was applied to an equal mixture of the diastereomers of ephedrine (Rice et al., 2018) and the equal mixture of the enantiomers of fluoxetine and the enantiomers of its metabolite norfluoxetine (unpublished, see Figure 2). For ephedrine, the mixture shows that concentration addition is a good approximation for the mixture of the diastereomers. The mixture of fluoxetine and norfluoxetine suggest a more synergistic effect, as the EC_{50} of 0.84 mg L^{-1} of the mixture is far lower than that of the individual compounds.



mg/L	Inhibition Results	
0.005 % inhib C5	20.6	0.1
0.050 % inhib C4	24.4	0.3
0.500 % inhib C3	12.5	0.2
5.000 % inhib C2	94.1	0.2
50.000 % inhib C1	102.0	0.0



mg/L	Inhibition Results			
0.05 % inhib C7	18.7	0.0	24hEC10 =	0.054
0.15 % inhib C6	3.9	0.1	24hEC20 =	0.107
5.00 % inhib C5	95.3	0.1	24hEC50 =	0.835
10.00 % inhib C4	95.0	0.0	24hEC70 =	3.290
16.00 % inhib C3	92.3	0.0	24hEC90 =	12.963
33.00 % inhib C2	97.3	0.0		
50.00 % inhib C1	100.5	0.1		

Figure 2 Ecotoxicity test of a racemic mixture of fluoxetine and norfluoxetine with *T. thermophila*

3.3. Development of new nanotest method

3.3.1. Nanotest development

The main limitation of the practicality of the standard toxicity test was the quantity of toxicant required. For example:

- For a range finding test, 10 mL is required of highest concentration to both test this concentration and prepare the other concentrations in the dilution series e.g. 100 mg L^{-1} would require 1 mg of toxicant.

- For the definitive test, 15.6 mL, or 20 mL for accurate preparation using a volumetric flask, is required for the highest concentration and preparation of the remaining concentrations e.g. 100 mg L^{-1} would require 2 mg of toxicant.

Therefore, to carry out 1 range finding test and duplicate the definitive test to ensure reproducibility, a minimum of 5 mg is required for each toxicant. Whilst this may appear to be a relatively small quantity, many toxicants are very expensive, not available in powder form or in quantities larger than 1 mg e.g. illicit drugs, enantiomers, and metabolites. Therefore, the initial aim of the nanotest development was to reduce the quantity of toxicant required, ultimately to increase the range of toxicants that can be tested.

24, 48 and 96-well plates were considered. The maximum working volume for the well plates were generally 1.0 mL, 0.5 mL, and 0.20 mL respectively. This would result in a population of (based on using the same proportion of protozoa inoculate to total volume as used in the standard test (40 µL ciliate inoculum in 2.0 mL test solution, resulting in ~100 protozoa per 1.0 mL)) of approximately 100, 50 and 20 protozoa in the final test cell. A population of only 20 protozoa was deemed far too small for a population-based inhibition test on a microorganism. Therefore, both 24-well and 48-well plates were considered further.

The following plate plans (Figures 3-5) were devised where C0 is the control and C1-C5 are the concentrations as planned in the standard test.

C0	C0	C0	C0	C0	C0
C0	C1	C2	C3	C4	C5
C0	C1’	C2’	C3’	C4’	C5’
C0	C0	C0	C0	C0	C0

Figure 3 24-well plate plan, where C0 is the control and C1 – C5 are the concentrations as planned in the standard toxicity test. C1’ – C5’ are the replicates

C0	C0	C0	C0	C0	C0	C0	C0
C0	aC1	aC2	aC3	aC4	aC5	aC6	C0
C0	aC1’	aC2’	aC3’	aC4’	aC5’	aC6’	C0
C0	bC1	bC2	bC3	bC4	bC5	bC6	C0
C0	bC1’	bC2’	bC3’	bC4’	bC5’	bC6’	C0
C0	C0	C0	C0	C0	C0	C0	C0

Figure 4 48-well plate plan, where C0 is the control, aC1 – aC6 are a range of 6 concentrations for toxicant ‘a’, bC1 – bC6 are a range of 6 concentrations for toxicant ‘b’, aC1’ – aC6’ and bC1’ – bC6’ are the replicates

The wells around the plate perimeter are most at risk from temperature fluctuations, so these wells were made controls, as the relatively large number of them will enable any outliers to be removed and provide a relevant average control response. From these two plans, the 48-well plate shows clear advantages of lower volumes, more controls, a larger range of concentrations and the space to test more than one toxicant at a time. Therefore the 48-well plate plan was progressed.

For coloured toxicants, such as the references chemical K_2Cr_2O7, a further test plate was devised (see Figure 5), based on the original standard protocol. This was to accommodate how the colour of the chemical may influence the OD measurement and it might change over the test period.

C0	C0	C0	C0	C0	C0	C0	C0
C0	C0	C1	C2	C3	C4	C5	C0
C0	C0	C1’	C2’	C3’	C4’	C5’	C0
C0	C0	C0	C0	C0	C0	C0	C0
C0	C0	C1x	C2x	C3x	C4x	C5x	C0
C0	C0	C0	C0	C0	C0	C0	C0

Figure 5 48-well plate plan for reference test with K_2Cr_2O7, or coloured toxicant, where C0 is the control, C1 – C5 are a range of 5 concentrations for the reference standard, C1’ – C5’ are the replicates and C1x - C5x are wells filled only with the reference standard solution at the respective concentrations i.e. no food or protozoa.

Overall, the application of this test highlighted several issues: shaking, test length and condensation. Automatic shaking was enabled to ensure a homogenous mixture prior to OD measurement however, this resulted in a shift of the plate and subsequent inaccuracy of measurements. Therefore, the shaking function was disabled.

Initially, it took far longer than the original 24 hours for the protozoa to clear 60% of the food in the well. This was thought to be due to one of two factors; temperature or population. The protozoa are sensitive to small changes in temperature. Therefore, increasing the temperature to 32 ˚C reduced the test time by several hours. Variations in population were also tested; increasing the population by a factor of 2 and 3 showed increasing stability in the first 16 hours of the test and a greater difference between the maximum OD and minimum OD of the test. These changes ensured the controls began passing the validation criteria of a 60% reduction in OD within 24 hours.

Condensation on the test plate lid became a considerable problem, leading to drastic changes in OD throughout the test. Conducting the test without a lid caused high levels of evaporation of the test medium and resulted in an inaccurate test of toxicity. Therefore, optical adhesive films were used instead of lids. Despite that this film also built up a layer of condensation, small holes could be carefully pricked though the optical film over each well, to prevent condensation and limit evaporation. 1, 5 and 9 holes were tested as shown in Figure 6. Overall, 9 holes were found to be the most effective at minimising condensation and appear to have a negligible effect of the volume of the well after 24 hours.

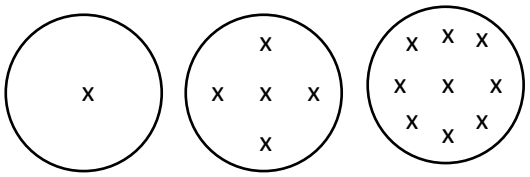


Figure 6 The distribution of holes over a well, 'x' in the test wells, 1, 5 and 9 holes respectively.

3.3.2. **Optimised nanotest protocol**

The final well test plate plan included positive control, using a concentration of 100 mg L^{-1} of $K_2Cr_2O_7$, and four negative controls, duplicates of food and synthetic freshwater (no protozoa) to show no variation over 24 hours without protozoa, and duplicates of protozoa only and synthetic freshwater (no food) to show lack of response without food. These plans can be seen in Figure 7. Furthermore, a seventh concentration was added.

With this optimised nanotest, the primary aim of reducing the mass of toxicant required has been achieved.

- For a range finding test, 1.5 mL is required of highest concentration to test this concentration and prepare the other concentrations in the dilution series e.g. 100 mg L^{-1} would require 0.15 mg of toxicant.

- For the definitive test, less than 5 mL is required for the highest concentration and preparation of seven concentrations e.g. 100 mg L^{-1} would require 0.5 mg of toxicant.

The results of this nanotest were reproducible. However, the 24h EC_{50} (17.0 mg L^{-1}) showed a reduction in the toxicity of the reference material compared with the reference values from the batch specification sheet (8.2 mg L^{-1} with an acceptable range of 5.3 – 10.5 mg L^{-1}). This is likely due to the change in ratio of population:food:test medium.

Future work to validate the test should explore the different ratio of population:food:test medium, to further understand the subsequent effects. Furthermore, the number of protozoa present in each well is currently determined theoretically. Tests should be carried out to determine how many protozoa are in each well, the standard variation during the preparation of the tests, and what resultant effect this has.

	1	2	3	4	5	6	7	8
A	C0	C0	C0	C0	C0	C0	C0	C0
B	C0	C1	C2	C3	C4	C5	C6	C7
C	C0	$C1^1$	$C2^1$	$C3^1$	$C4^1$	$C5^1$	$C6^1$	$C7^1$
D	C0	C0	C0	C0	C0	C0	C0	C0
E	C0	C1x	C2x	C3x	C4x	C5x	C6x	C7x
F	C0	Positive control	Food only	Food only	Protozoa only	Protozoa only	C0	C0

	1	2	3	4	5	6	7	8
A	C0	C0	C0	C0	C0	C0	C0	C0
B	C0	C1	C2	C3	C4	C5	C6	C7
C	C0	$C1^1$	$C2^1$	$C3^1$	$C4^1$	$C5^1$	$C6^1$	$C7^1$
D	C0	C1	C2	C3	C4	C5	C6	C7
E	C0	$C1^1$	$C2^1$	$C3^1$	$C4^1$	$C5^1$	$C6^1$	$C7^1$
F	C0	Positive control	Food only	Food only	Protozoa only	Protozoa only	C0	C0

Figure 7 Final 48 well plate plans, top shows reference/coloured toxicant test plate and the bottom shows a two toxicant test plate.

The optimised nanotest protocol is as follows:

1) Preparation of toxicant dilution series

i. *Range finding test*

Prepare a stock solution of the toxicant that should theoretically show 100% inhibition (C1). From this prepare a 1:10 dilution series spanning 7 orders of magnitude, with 1.5 mL in each eppendorf. This is carried out by transferring 0.15 mL of C1 and 1.35 mL of synthetic freshwater to a test tube labelled C2. Mix thoroughly. Then transfer 0.15 mL of C2 and 1.35 mL of synthetic freshwater to a test tube labelled C3 and mixed this thoroughly. Continue this process until there are 7 concentrations.

ii. *Definitive test*

From the results of the range finding test, 80-100% inhibition of population growth and 0-20% inhibition growth should be determined. If not, then the test should be repeated for a more appropriate range until these concentrations are found. The definitive test is prepared from the lowest concentration which causes 80 – 100% inhibition (this will be C1 in the definitive test) and the highest concentration which causes less than 20% inhibition (this will be C7). The remaining concentrations (C2-C6) are prepared to cover the range between these two concentrations. The range of concentrations should be; C2 = 0.56 x C1, C3 = 0.32 x C1, C4 = 0.18 x C1, C5 = 0.10 x C1, C6 = 0.03 x C1 and C7 = 0.01 x C1. Use synthetic freshwater to dilute.

2) Preparation of ciliate inoculum

Carefully shake the stock *T. thermophila* culture vial to mix, then using a sterile syringe, extract 500 µL from the vial and transfer to semi-macro cuvette. Add 1.0 mL of deionised water. Cap the cuvette and shake gently to mix. Using the spectrophotometer at 440 nm wavelength, measure the OD of the vial. Calculate the amount of deionised water required to reach a theoretical OD value of 0.040, using equation 1 and 2. Transfer 500 µL of the inoculum in the semi-macro cuvette to the ciliate inoculum tube and add the dilution volume determined from equation 2. Cap the tube and mix thoroughly. This is important to regulate the ciliate population, as this will result in approximately 100 protozoan per mL in each of the final test cells (MicroBioTests, n.d.).

3) Preparation of the food substrate

Transfer one vial of reconstitution medium to the food substrate vial, close the vial and mix thoroughly.

4) Inoculation of the test cells

Following the relevant plate plan, add 0.5 mL of synthetic freshwater to the wells labelled C0. To the remaining wells, add 0.5 mL of each of the 7 concentrations to the corresponding wells. To each well, add 10 µL of food substrate and 20 µL of the prepared ciliate inoculum. In each well, after the addition of the inoculum, draw 20 µL,

3 times, the inoculated test solution into the pipette and release back into the cell to mix and to ensure no protozoa remain in the tip. Make sure to use a new tip for each well.

5) Seal plate

Cover plate with optical adhesive film. Carefully pierce 9 holes in the film above each well as illustrated in Figure 6, being careful not to touch the test medium within each well.

6) OD measurements and incubation of test plate

Place the plate into pre-heated plate reader at 32˚C. Close plate drawer. Set plate reader to take an OD measurement at 440 nm wavelength, every 5 minutes for 28 hours, with no shaking.

7) Data processing

Export raw data to Excel. Calculate change in optical density between T0h and T24h. Input this into the standard tox kit software to calculate the 24 h EC_{50} (also EC_{10}, EC_{20}, EC_{70} and EC_{90}).

This test protocol not only uses less toxicant but also allows more than one toxicant to be easily prepared and tested in parallel, reducing overall experiment time. Furthermore, the range of kinetic data may be of use. Previous tests only had two OD measurements, once at the beginning and once at the end. With this method, trends across 24 hours can be seen.

4. Preliminary conclusions and future work

During ecotoxicity testing using a standard protocol, a variety of test organisms indicated some are more sensitive to varying stereochemistry. *T. thermophila* has been shown to be particularly sensitive to this (Andrés-Costa et al., 2017; Rice et al., 2018). Furthermore, this unusual test organism can be found in both the aquatic and terrestrial environment, as well as within WwTW processes.

Standard toxicity tests are inefficient in their use of toxicants, this can be minimised for some test organisms, i.e. microorganisms. This work shows the development of a new nanotest using the ciliate protozoa *T. thermophila*, adapted from the standard Protoxkit toxicity test produced by MicroBioTests. The validation of this nanotest is on-going but looks to be a promising addition to the ecotoxicity

toolbox. It not only uses less toxicant, but also enables testing of multiple toxicants on one plate. Firstly, this is particularly beneficial when studying different enantiomers, as pure enantiomers are often far more expensive than their racemic mixture of the compound. Therefore, the ability of using smaller quantities in the tests is financially and logistically beneficial. Secondly, the ability to test multiple toxicants on one plate will allow both enantiomers to be tested at the same time, reducing any potential unintended variation from carrying out the tests at different times. Furthermore, the SpectraMax M3-R plate reader enabled continuous kinetic data acquisition, therefore not only allowing determination of population growth by measuring the reduction of the OD, but also allowing assessment of the curve characteristics as an additional quality control measure.

Overall this work shows significant differences in the ecotoxicity of stereoisomers of CECs, particularly pharmaceuticals. However, this is overlooked in current ERA guidelines. Inaccuracies in ERAs for chiral CECs is therefore highly likely, as MECs determined via achiral analysis provide no information on the EF and therefore the actual potency of the mixture cannot be determined. More investigation and further development of efficient toxicity tests would be highly beneficial to building up a comprehensive database of stereochemical ecotoxicity, which has been identified as highly lacking.

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Appendix 6B: Statement of Authorship

This declaration concerns the article entitled:			
Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance			
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [X]			
Publication details (reference)	Andrés-Costa MJ, Proctor K, Sabatini MT, Gee AP, Lewis SE, Pico Y, et al. Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance. Sci Rep. 2017;7(1).		
Copyright status (tick the appropriate statement) I hold the copyright for this material [X] Copyright is retained by the publisher, but I have been given permission to replicate the material here [ ]			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: The candidate was the minor contributor in the formulation of the original idea. Design of methodology: The candidate considerably contributed to the design of the study Experimental work: The candidate the major contributor in the use of standard toxicity tests, predominantly the *Tetrahymena thermophila* test, and contributed to the preparation and collection of microcosm samples. Presentation of data in journal format: The candidate was the major contributor for the data analysis, interpretation, and presentation of the data of the *T. thermophila* test and assisted in the writing of the paper.		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	[redacted]	**Date**	11/12/2020

Published Paper

Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance

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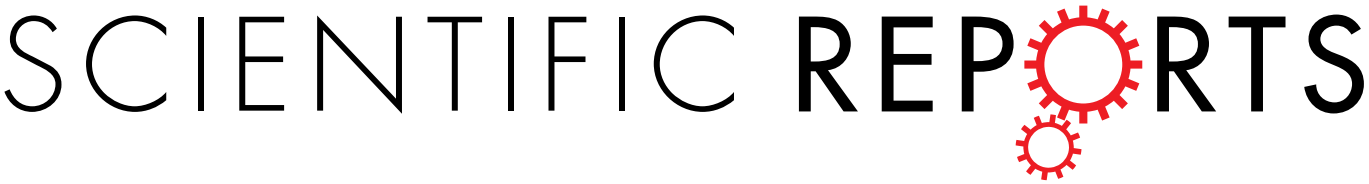

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Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance

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European legislation focusing on water quality is expected to broaden to encompass several pharmaceuticals as priority hazardous substances. This manuscript aims to challenge current regulatory approaches that do not recognize stereochemistry of chiral pharmaceuticals by testing the hypothesis that environmental transformation and effects of chiral pharmaceuticals are stereoselective. Our experiments revealed that, while degradation of chiral fluoxetine (FL) in river water occurs via non-enantioselective photochemical and mildly-enantioselective microbial processes favoring the (*R*)-enantiomer, a pronounced enantioselectivity favoring (*S*)-FL (leading to the formation of (*S*)-NFL (norfluoxetine)) is observed during activated sludge treatment. Toxicity tests proved strong enantiomer-specific toxicity in the case of *Tetrahymena thermophila*, protozoa that are utilized during activated sludge treatment ((*R*)-FL is 30× more toxic than (*S*)-FL; (*S*)-NFL is 10× more toxic than (*S*)-FL). This is of paramount importance as preferential degradation of (*S*)-FL in activated sludge microcosms leads to the enrichment of FL with 30× more toxic (*R*)-FL and formation of 10× more toxic (S)-NFL. It is commonly assumed that a decreased concentration of FL leads to decreased biological impact. Our study proves that despite the overall decrease in FL concentration, accumulation of toxic (*R*)-FL and formation of toxic (*S*)-NFL leads to much higher than presumed toxicological effects.

Pharmaceuticals are a group of pollutants with growing evidence regarding their environmental impacts. European legislation focusing on water quality is expected to broaden to encompass several pharmaceuticals as priority hazardous substances. This manuscript challenges current regulatory approaches that do not recognize stereochemistry of chiral pharmaceuticals. Fluoxetine (FL, known as Prozac) is used here as an example.

FL is a diphenhydramine derivative and selective serotonin reuptake inhibitor (SSRI). It is used to treat a variety of mental health problems such as depression, panic, anxiety, or obsessive-compulsive symptoms. There was a 165% increase in the prescribing of antidepressant drugs in England between 1998 and 2012 (an average of 7.2% per year)[1]. Indeed, FL is the fourth most prescribed antidepressant in England, and accounts for 11.3% of all antidepressant drug use[2].

FL is extensively metabolized to norfluoxetine (NFL) and several other metabolites such as FL glucuronide, NFL glucuronide, *para*-trifluoromethylphenol and hippuric acid. The principal metabolite, NFL, is formed by *N*-demethylation of FL. The potency and selectivity of NFL's SSRI activity is similar to that of the parent drug. The elimination of FL accounts for 80% excreted in the urine (as 11.6% FL, 7.4% FL glucuronide, 6.8% NFL, 8.2% NFL glucuronide, >20% hippuric acid, 46% other) and approximately 15% excreted in the feces[3].

Recent research studies have shown that most pharmaceuticals, including FL and NFL, enter the aquatic environment via (un)treated communal wastewater. Both FL and NFL have been detected in wastewater and receiving waters at levels ranging from ng L^{-1} to μg L^{-1} [4–14]. Furthermore, they were found in the tissue of fish collected near municipal wastewater discharges. Both FL and NFL remain biochemically active in the environment and can have marked effects on the morphology, physiology, and behavior of different species[6,10,15–18].

Despite some limited research on fate and effects of FL, there has been very little attention paid to the stereochemistry of FL and its possible environmental impacts. FL has one chiral carbon in its structure and as a result it exists in two enantiomeric forms as (*S*)-FL and (*R*)-FL. Similarly, NFL exists in two enantiomeric forms as

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(*S*)-NFL and (*R*)-NFL. Enantiomers of the same drug have identical physicochemical properties but may differ in their biological properties. Thus, chiral drugs can undergo stereoselective mechanisms controlling their fate such as distribution, metabolism and excretion, as these processes (due to stereoselective interactions of enantiomers with biological systems) usually favor one enantiomer over the other. This leads to process-dependent changes in the enantiomeric composition of chiral compounds[9]. Metabolism of FL was found to be enantioselective in humans, with the (*R*)-enantiomer being metabolized faster than (*S*)-enantiomer[19]. Additionally, due to different pharmacological activity, enantiomers of chiral drugs can differ in their biological actions, potency and toxicity[20]. Enantiomers of FL have similar potency as inhibitors of the serotonin reuptake pump in humans whereas enantiomers of NFL act differently, with (*S*)-NFL showing higher inhibition capacity[21].

Ecotoxicity of FL (and other pharmaceuticals) is currently assessed for the racemate, as FL is marketed as a racemic mixture of two enantiomers. Environmental risk assessment (ERA) approaches need to be re-evaluated as they are based on a simplistic assumption that FL present in the environment is racemic[9]. Indeed, limited research indicates that FL and NFL are present in the aqueous environment as non-racemic mixtures, i.e. enriched with one enantiomer[5,22–26]. Furthermore, FL was found to undergo enantioselective transformation during wastewater treatment[5,27]. In a recent study, Barclay *et al*. (2011) found a slight enrichment of FL and NFL with (*S*)-enantiomer in both raw and treated wastewater[22]. In contrast, MacLeod *et al*. (2007) reported that in their monitoring study FL was enriched with (*R*)-enantiomer in wastewater indicating faster degradation of (*S*)-enantiomer during wastewater treatment[5,23]. Ribeiro *et al*.[28] did not observe enantioselectivity in fluoxetine's biodegradation in activated sludge. However, the same group observed enantioselective degradation favouring (*R*)-FL by Labrys portucalensis strain F11[29].

FL is often used as a model compound for assessing SSRI impact on aquatic organisms such as zebrafish, Japanese medaka, goldfish, gulf toadfish, rainbow trout, fathead minnows and polychaete worms (*Capitella teleta*)[30–36]. FL was reported as toxic at low concentrations to several aquatic species[30,37–39] but enantiomer-dependent toxicity was not considered. In fact, FL was proposed as one of 10 pharmaceuticals potentially dangerous for the environment[5]. Enantioselective toxicity of FL was demonstrated for *Primephales promelas* and *Tetrahymena thermophila*, where (*S*)-FL was found to be more toxic than its respective enantiomer[34,38]. On the other hand (*R*)-FL was considered more harmful to *Pseudokirchneriella subcapitata*[38]. To the authors' knowledge, there are no published reports on NFL toxicity to aquatic organisms at enantiomeric level. NFL was reported to be more active in humans than the parent compound[40]. Fuller *et al*.[21] determined that enantiomers of NFL have markedly different potencies as inhibitors of the uptake of serotonin with (*S*)-NFL being more potent than the (*R*)-enantiomer in rats. It is therefore expected that NFL's ecotoxicity to aquatic organisms might also be enantiomer-dependent.

The above discussion clearly indicates that current ERA approaches that do not recognize stereochemistry of chiral pharmaceuticals are inaccurate and could lead to incorrect conclusions being drawn regarding the ecotoxicological effects of chiral drugs. The limited work in the area of stereochemistry-induced fate and effects of FL (and pharmaceuticals in general) is mainly due to lack of enantioselective analytical methods as well as availability of (affordable) enantiomerically pure analytical standards. Such analytical methods and affordable enantiomerically pure standards are essential to gather accurate data needed for comprehensive ERA of these compounds.

The overarching aim of this study was to verify, for the first time, enantiomer-dependent fate and ecotoxicological effects of FL and its main metabolite NFL in the aquatic environment. To achieve the aim, the key components of this study were:

i) To synthesize single enantiomers of FL and NFL.
ii) To develop an analytical method for the detection and quantification of enantiomers of FL and NFL utilizing chiral liquid chromatography coupled with tandem mass spectrometry.
iii) To undertake, for the first time, mechanistic study of the degradation of FL and NFL formation in controlled river water and activated sludge simulating microcosm experiments.
iv) To verify, for the first time, enantiomer-specific toxicity of FL and NFL in aquatic species.

Results

Synthesis of enantiomerically pure FL and NFL enantiomers. Lack of commercially available and affordable enantiomerically pure standards is the key factor hindering progress in the understanding of bio-physicochemical processes governing the fate and effects of chiral pharmaceuticals and the resulting environmental risks. To overcome this limitation and to enable toxicity studies on single-enantiomer drugs, we propose a conceptually straightforward approach. For any given drug of interest, our approach relies on taking a known chemical synthesis of the racemic form (for example, from the patent literature), and carrying out the same procedure, yet employing a single enantiomer of starting material. This removes the need to develop new synthetic procedures and allows for the rapid and cost-effective production of single enantiomer drug substance on a scale sufficient to enable toxicity studies. Careful choice must be given to the selection of the most appropriate literature synthesis, since the concept is not universally applicable – in some instances, a synthetic procedure might induce unwanted racemisation of one of the synthetic intermediates en route to the final drug substance. Nevertheless, in the case of FL and NFL, we have established a synthetic protocol that allows access to single enantiomers of NFL and FL, in just 3 or 4 synthetic steps, respectively. The single enantiomer starting material required (**1**) is commercially available in both the (*R*) and (*S*) forms (99% *e.e.*), and the cost is not prohibitive. Our protocol draws on multiple literature sources, as shown in Fig. 1. Thus, from single enantiomer starting material **1**, as per one of the original patents on fluoxetine[41], reaction with potassium phthalimide affords single enantiomer **2**, which in turn undergoes hydrazinolysis to give single enantiomer **3**. Primary amine **3** can then be employed in a S_NAr reaction to give single enantiomer NFL. Various synthetic procedures are reported for the conversion of NFL into FL; we

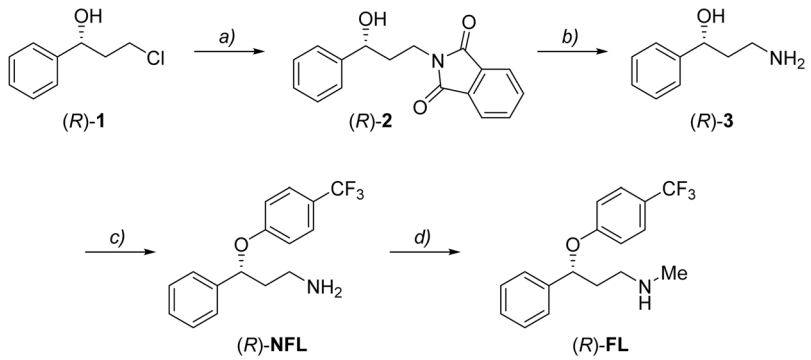

Figure 1. Synthesis of single enantiomers of NFL and FL. *Reagents and conditions*: (**a**) 1.2 equiv. potassium phthalimide, DMF, 90 °C, 2 h, 88%. (**b**) 3 equiv. $NH_2NH_2{\cdot}H_2O$, ethanol (EtOH), reflux, 2 h, 85%. (**c**) 1.5 equiv. sodium hydride, 1 equiv. *p*-fluorobenzotrifluoride, DMSO, 1 h, 90 °C, 71%. (**d**) 1.1 equiv. methyl chloroformate, 5 equiv potassium carbonate, CH_2Cl_2/H_2O 1:2, 20 min, room temp., *then* 2 equiv. lithium aluminium hydride, THF, 2 h, reflux, 80%.

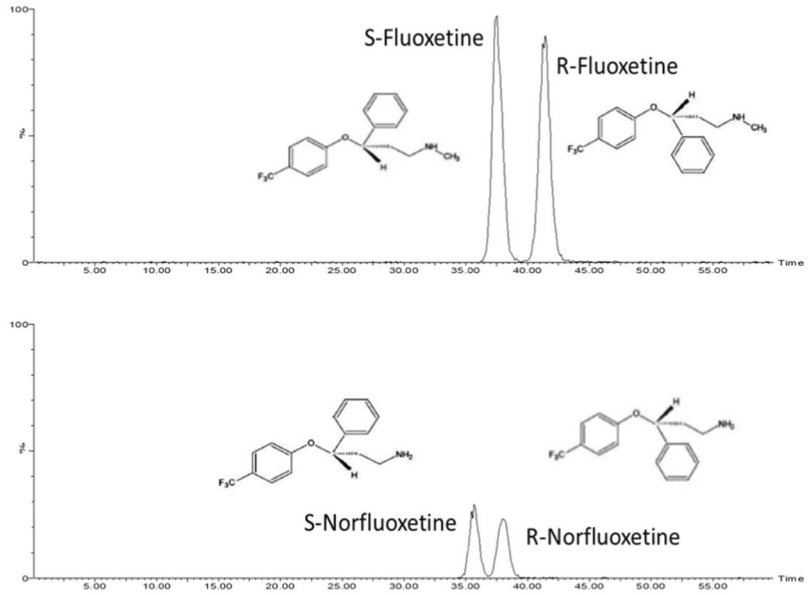

Figure 2. Chromatographic separation of enantiomers of FL and NFL.

opted to employ carbamate formation followed by hydride reduction to convert a proportion of our synthesized NFL into FL[42]. By the above means we were able rapidly to access the required quantities of single enantiomer material to carry out the studies detailed below.

Enantioseparation of FL and NFL enantiomers with chiral LC-MS/MS. Unavailability of analytical methods allowing for enantiomeric separations of trace concentrations of chiral drugs in complex environmental matrices is a limiting factor hindering progress in fundamental understanding of their fate and effects. We therefore developed a robust, sensitive and selective method utilizing chiral liquid chromatography coupled with tandem mass spectrometry for enantiomeric separation of FL and NFL. We used Astec Chirobiotic V (CBV) column with mobile phase (pH 6.5, 0.06 mL min^{-1}, 25 °C) composed of 70% of ethanol (EtOH), 30% of ultra-pure water (HQ water), 4 mM of ammonium acetate (AAC) and 0.005% of formic acid (FA) under isocratic conditions to achieve baseline separation of enantiomeric pairs (Fig. 2, R_s (resolution of enantiomers) = 1.41 and 1.00 for FL and NFL, respectively). All conditions tested and results can be found in Table S1 in the supplementary information section. The method showed good linearity ($R^2 > 0.99$) for all four enantiomers within the studied range (0.5–100 µg L^{-1}). Method detection and quantification limits (MDLs and MQLs) for river water matrices ranged from 1.2 to 1.3 ng L^{-1} and from 4.6 to 5.1 ng L^{-1}, respectively. In the case of activated sludge matrices, MDLs ranged from 0.4 to 0.8 ng L^{-1} and MQLs ranged from 1.7 to 3.1 ng L^{-1} (Table S2). The accuracy and precision were within ±20% (Table S3). Very good recoveries accounting for >67% were observed in the case of all four enantiomers in all studied matrices. Matrix effect (ME) accounted for <15.6%.

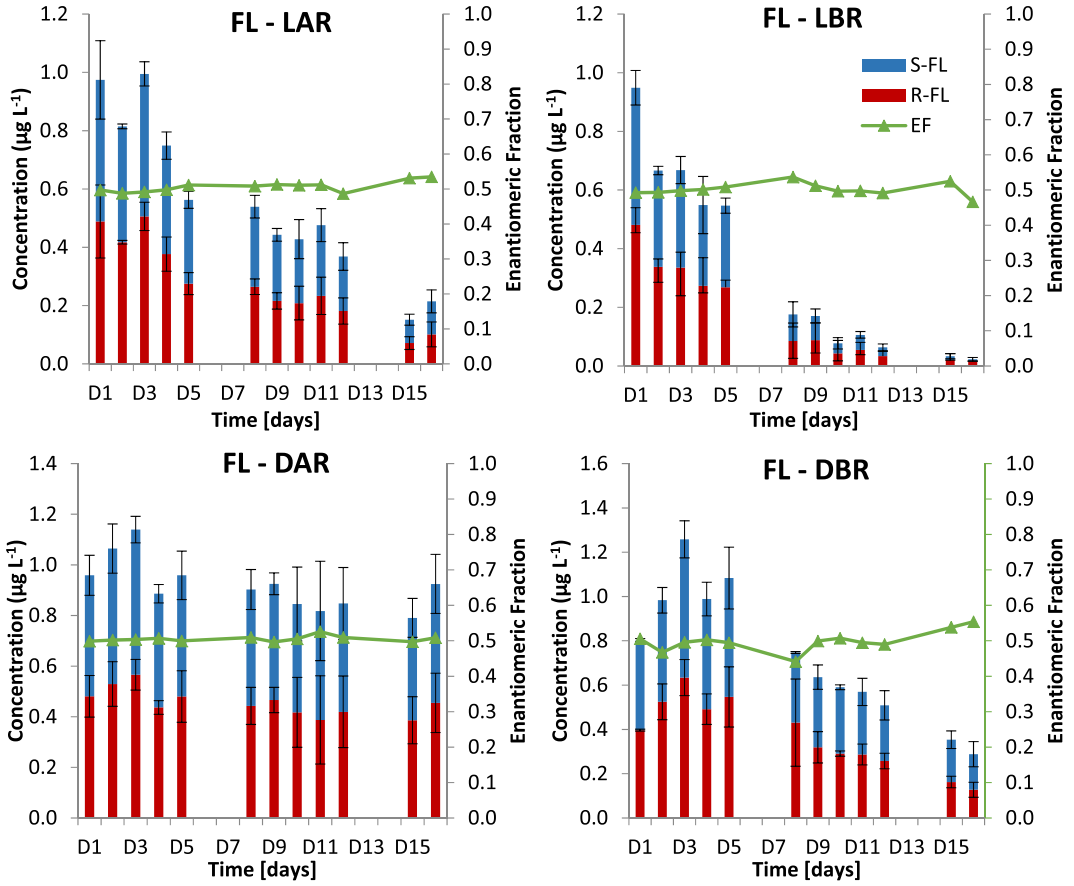

Figure 3. Degradation of FL in river water simulating microcosms under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols).

Synthesized enantiomerically pure FL and developed chiral LC-MS/MS method were used to verify transformation of FL in river and activated sludge simulating microcosms and their ecoxoticological impacts.

Transformation of FL and NFL in river and activated sludge simulating microcosms. *River water microcosms.* The river simulating microcosms revealed that degradation of FL takes place via both microbial and photochemical processes (Fig. 3 and Table S4). Photolysis is considered to be the most important phenomenon contributing to the degradation of FL, as 74.5% (*S*)-FL and 79.2% (*R*)-FL of FL were removed in light abiotic conditions (LAR). This process, as expected, was found not to be enantioselective. Microbial processes resulted in mild enantioselectivity towards (*R*)-enantiomer and led to the removal of 60.4% of (*S*)-FL and 67.9% of (*R*)-FL at dark biotic conditions (DBR). As expected, the light biotic reactor (LBR) utilizing both photochemical and microbial processes led to the highest removal of FL: 98.4% of (*S*)-FL and 96.7% of (*R*)-FL. Dark abiotic conditions (DAR) did not lead to any significant removal of FL. Traces of NFL were observed in both abiotic and biotic conditions (Figure S1). This indicates that degradation of FL leading to NFL formation takes place as a result of both photochemical and microbial processes.

Activated sludge microcosm. Transformation of FL in activated sludge simulating microcosms was studied at two concentration levels: 10 and 100 µg L^{-1} of racemic FL (Fig. 4 and Table S5). In both cases a significant decrease in the concentration of (*S*)-FL and (*R*)-FL was observed. In the microcosm spiked with 10 µg L^{-1} rapid removal of FL occurred during the first 30 minutes (50% degradation). This process was not stereoselective (enantiomeric fraction (EF) 0.5) and did not lead to expected formation of NFL. It is therefore postulated that this high rapid removal of FL from the aqueous phase during the first 30 min of the experiment is due to its sorption to suspended particulate matter. Further removal of FL in 10 µg L^{-1} reactor was much slower and led to its stereoselective transformation favoring (*S*)-FL (EF < 0.3) and leading to the formation of NFL enriched with (*S*)-enantiomer (EF > 0.7). As the activated sludge simulating microcosms were undertaken in the dark, it is postulated that observed stereoselective transformation of FL and stereoselective formation of NFL is due to the prevalence of stereoselective microbial metabolic processes in studied bioreactors. Molar percentage yield of NFL formation denoted: 10.7% and 6.2% for (*S*)-FL and (*R*)-FL, respectively.

Similar observations were recorded in the microcosm spiked with 100 µg L^{-1} of FL. However, the effect of sorption was not observed. This is probably due to much higher initial FL load in 100 µg L^{-1} bioreactor not allowing for the change to be recorded. Stereoselective microbial processes resulted in 60% transformation of FL with

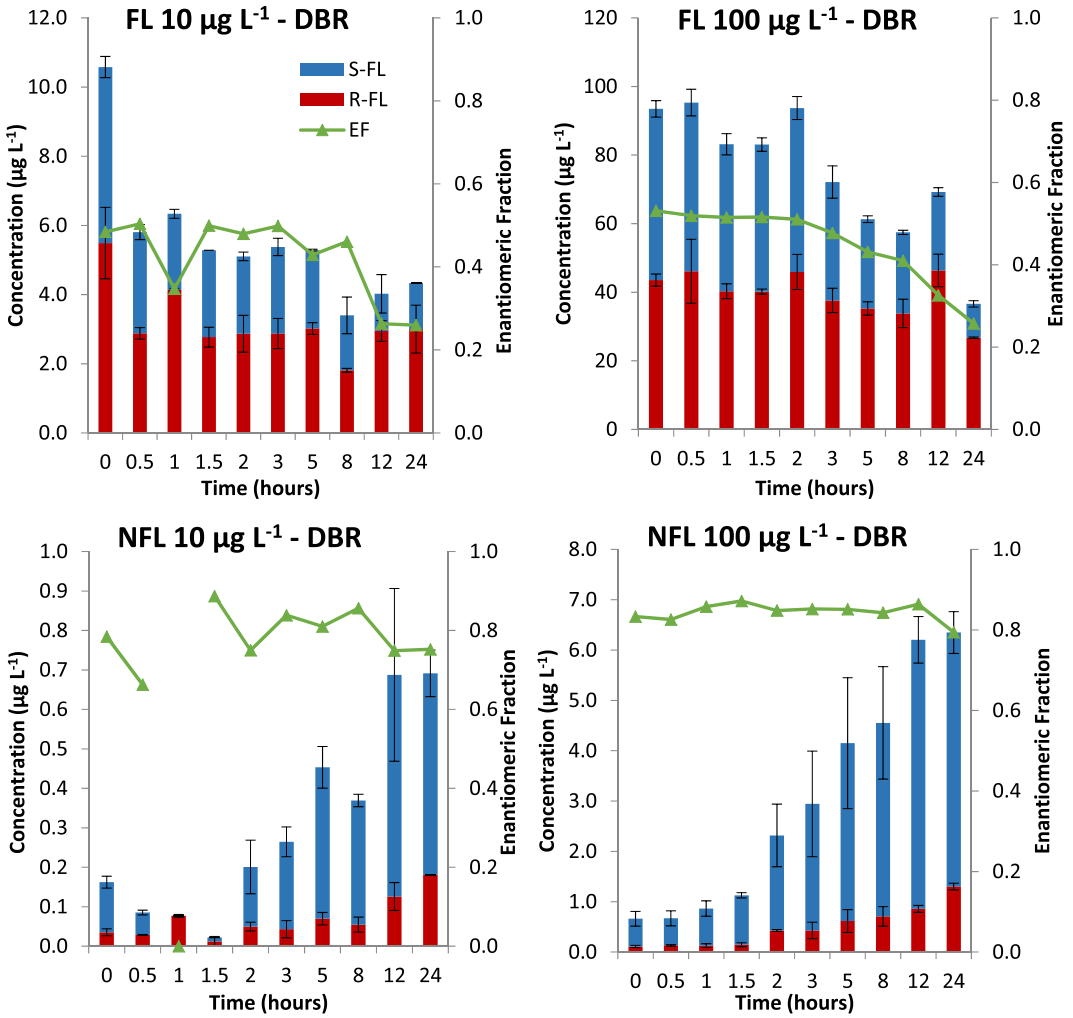

Figure 4. Degradation of FL and formation of NFL in activated sludge simulating microcosms under dark biotic (DBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols).

		R^2	SS [g L^{-1}]	k_1 [h^{-1}]	$t_{1/2}$ [h]	k_{biol} [$Lg_{SS}^{-1}h^{-1}$]	$t_{1/2biol}$ [h]
10 µg L^{-1}							
(*S*)-FL	y = −0.0715x - 0.0584	0.8696	2.0	0.07	9.69	0.04	18.9
(*R*)-FL	No degradation	n/a	n/a	n/a	n/a	n/a	n/a
100 µg L^{-1}							
(*S*)-FL	y = −0.0721x - 0.0667	0.8216	2.0	0.07	9.61	0.04	19.4
(*R*)-FL	y = −0.0206x - 0.0274	0.8202	2.0	0.02	33.6	0.01	68.0

Table 1. Degradation pseudo-first order rate constants (k_1 and k_{biol}) in single-compound activated sludge simulating microcosm. n/a - not calculated due to no degradation of (*R*)-(−)-enantiomer.

twice as high preference towards (*S*)-FL (EF < 0.3, 80% removal of (*S*)-FL and only 38% removal of (*R*)-FL) and formation of NFL enriched with (*S*)-enantiomer (EF > 0.7). Molar percentage yield of NFL formation denoted: 11.7% and 7.4% for (*S*)-FL and (*R*)-FL. This is in agreement with results obtained for 10 µg L^{-1} bioreactor. Interestingly in both bioreactors, long lag phases (3 h and 2 h in the case of 10 µg L^{-1} and 100 µg L^{-1} FL bioreactor, respectively) were observed.

Kinetic studies (Table 1) confirmed low biodegradation of FL and the more recalcitrant nature of (*R*)-FL. k_{biol} and $t_{1/2biol}$ of (*S*)-FL transformation were 0.04 $Lg_{SS}^{-1}h^{-1}$ and 19 h respectively in both 10 and 100 µg L^{-1} bioreactors. k_{biol} and $t_{1/2biol}$ of (*R*)-FL transformation were much lower and denoted 0.01 $Lg_{SS}^{-1}h^{-1}$ and 68 h respectively in 100 µg L^{-1} bioreactors. Due to the lack of degradation of (*R*)-FL in 10 µg L^{-1} bioreactor, no kinetic studies could be undertaken.

Organism	Test	Toxicity endpoints	Effect [mg L^{-1}]				Ref
			FL		NFL		
			(*S*)-	(*R*)-	(*S*)-	(*R*)-	
P. promelas	$LOEC_{7d}$	-survival	0.10	0.17	n/a	n/a	34
		-growth	0.05	0.17	n/a	n/a	
		-feeding	0.05	0.17	n/a	n/a	
	$LC50_{48h}$	-survival	0.22	0.21	n/a	n/a	34
D. magna	$LOEC_{21d}$	-immobilization	0.44	0.43	n/a	n/a	34
		-reproduction	0.44	0.43	n/a	n/a	
		-grazing	0.20	none	n/a	n/a	
	$LC50_{48h}$	-immobilization	6.9	8.1	n/a	n/a	38
	$EC50_{48h}$	-immobilization	3.6	4.1	2.8	2.9	(this study)
T. thermophila	$EC50_{24h}$	-growth	3.2	30.5	n/a	n/a	38
	$EC50_{24h}$	-growth	35.2	1.3	3.8	5.8	(this study)

Table 2. Ecotoxicity of FL and its metabolite NFL (n/a – not analysed).

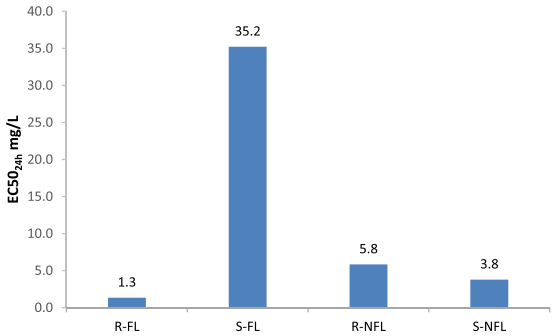

Figure 5. $EC50_{24h}$ for the *T. thermophila* test. See Tables S7–14 for CV% of individual tests.

The results above indicate that longer sludge retention times might be needed during wastewater treatment in order to facilitate degradation of FL. However, it should be noted that these processes are likely to be stereoselective and could potentially lead to enrichment of FL with the more potent enantiomer, as well as formation of biologically active metabolites; this is despite the nominal decrease in concentration levels of FL.

Ecotoxicity of FL and NFL. Enantiomer-dependent toxicity of FL and NFL was evaluated for two aquatic organisms: *Daphnia magna* and *Tetrahymena thermophila*. $EC50_{48h}$ for FL enantiomers towards *D. magna* was 3.6 mg L^{-1} and 4.1 mg L^{-1} for (*S*)-FL and (*R*)-FL respectively (Table 2). $EC50_{48h}$ for NFL towards *D. magna* denoted 2.8 mg L^{-1} and 2.9 mg L^{-1} for (*S*)-NFL and (*R*)-NFL (raw data are shown in Table S6). The results indicate a noticeable difference between the toxicity of FL and NFL, NFL being more toxic than FL, but no significant enantioselectivity was observed for studied enantiomeric pairs. In contrast, $EC50_{24h}$ for FL enantiomers towards *T. thermophila* was strongly enantiomer dependent and denoted 35.3 mg L^{-1} and 1.3 mg L^{-1} for (*S*)-FL and (*R*)-FL, respectively. These results contradict those published by De Andrés *et al.*[38] as within that study it was observed that the (*S*)-enantiomer was more toxic with an $EC50_{24h}$ of 3.2 mg L^{-1} compared to the (*R*)-enantiomer with an $EC50_{24h}$ 30.5 mg L^{-1}. To confirm the validity of measurement undertaken in this work, the stock solutions and the test cells were analyzed with the chiral-LC-MS/MS method. The results showed that the changes in the concentrations of the toxicants were minimal during the test, however they support the use of the correct enantiomer in this test. This was further confirmed by the use of enantiomerically pure analytical standards to confirm the retention time of each enantiomer (Figure S2).

Similarly to FL, $EC50_{24h}$ for NFL enantiomers towards *T. thermophila* was strongly enantiomer dependent. The $EC50_{24h}$ for NFL denoted 3.8 mg L^{-1} and 5.8 mg L^{-1} for (*S*)-NFL and (*R*)-NFL, respectively. Unlike FL, the (*S*)-NFL is more toxic than (*R*)-NFL. This is of paramount importance as preferential degradation of less toxic (*S*)-FL in activated sludge microcosms leads to the formation (and accumulation) of more toxic (*S*)-NFL. This is also an important consideration as *T. thermophila* is part of the microbial community of activated sludge ($EC50_{24h}$ data for *T. thermophila* is shown in Fig. 5 and Tables S7–S14).

Discussion

This report is, to the authors' knowledge, the first to study transformation of FL in environment simulating microcosms combined with ecotoxicological effects. The research reported in this manuscript tested and validated the hypothesis that degradation of FL, and formation of its main metabolite NFL, are enantioselective and biological in nature, and that their toxicity is enantiomer-dependent.

The river simulating microcosms revealed that degradation of FL takes place via both microbial and photochemical processes. Non-stereoselective photolysis was observed to be the most important phenomenon contributing to the degradation of FL. Microbial processes resulted in only mild enantioselectivity towards the (*R*)-enantiomer. However, a pronounced stereoselectivity was observed during activated sludge simulating microcosms. Microbial metabolic processes of FL during activated treatment process favored the (*S*)-enantiomer, which led to the enrichment of FL with the (*R*)-enantiomer. This is in contrast to metabolic processes in humans, which favor the (*R*)-enantiomer and lead to enrichment of FL in urine with the (*S*)-enantiomer[19]. The outcomes of human metabolism studies as well as full scale and microcosm wastewater treatment measurements indicate that enantiomeric signature of FL can change subject to composition and structure of microbial communities present in wastewater. Indeed in our full scale untreated wastewater study, FL was enriched with the (*S*)-enantiomer (EF 0.7)[43]. This confirms, yet again, complexity of environmental processes and reinforces the need for further comprehensive studies focusing on transformation of chiral pollutants in the environment.

Toxicity tests showed that while there is no significant enantioselectivity in the toxic response from *D. magna* to both FL and NFL, a strong enantiomer-dependent toxicity is observed in the case of *T. thermophila* ((*R*)-FL 30× higher than (*S*)-FL and (*S*)-NFL 10× higher than (*S*)-FL).

The above results indicate that traditional toxicological studies that do not recognize the importance of stereochemistry might not reveal the true toxicological impact resulting from stereochemistry of chiral drugs. Our research indicates that (*S*)-FL is preferentially degraded in activated sludge microcosms. This is expected, as (*S*)-FL is the least toxic to protozoa (organisms that are known to be key contributors to activated sludge treatment process) out of all four FL/NFL enantiomers studied. Unfortunately, this also indicates that FL, due to preferential metabolic degradation of (*S*)-FL, gets enriched with more toxic (*R*)-FL and leads to the formation of more toxic (*S*)-NFL. This accumulation of toxic (*R*)-FL and (*S*)-NFL will have detrimental effects on the performance of activated sludge treatment processes.

This study revealed that there are several, unaccounted for, underlying issues in both exposure and hazard assessment within ERA of chiral pharmaceuticals. One can assume that, if the stereochemistry of FL is not considered, decreased concentration of FL as a result of activated sludge treatment leads to decreased biological impact. Such an approach (as currently applied in ERA) can lead to false conclusions impacting environmental health. Our study proves that despite the overall decrease in FL concentration, accumulation of toxic (*R*)-FL and formation of toxic (*S*)-NFL in activated sludge will likely lead to higher toxicological effects, as observed in the case of protozoa. The European Medicines Agency guideline on the ERA of Medicinal Products for Human Use[44] and the EU Directive for ERA for Veterinary Medicinal Products[45] recommend the estimation of exposure and the prediction of risk calculation for the whole parent compounds only i.e. as a racemate or a mixture of stereoisomers if distributed as such. Therefore, current ERA leads to under or overestimation of toxicity of chiral pharmaceuticals and to incorrect ERA as chiral pharmaceuticals are present in the environment in their non-racemic forms and they show enantiomer-specific biological effects. We therefore recommend the adoption of a new strategy within ERA acknowledging stereochemistry of studied targets.

Methods

Chemicals and materials. HPLC-grade methanol (MeOH), EtOH, AAC, (99%), FA (98%) were purchased from Sigma Aldrich (Cambridge, UK). HQ water was supplied by a Milli-Q system (PURELAB, Elga, UK).

The reference standards, *rac*-FL and *rac*-NFL and the internal standard (IS) FL-d_5 were purchased from LGC Standards (Teddington, UK). All standards and ISs were of the highest purity available (>97%). Structures, molecular formulae and molecular weights of target enantiomers are summarized in Table S15.

Stock solutions of the individual compounds were purchased in MeOH at a concentration of 1 mg mL^{-1} or 0.1 mg mL^{-1} and stored in the dark at −16 °C. Working solutions were prepared by diluting stock solutions in mobile phase or MeOH on a daily basis and stored at 4 °C.

All glassware was deactivated with dimethyldichlorosilane (5% DMDCS in toluene, Sigma-Aldrich) to minimize sample loss through adsorption of basic analytes onto –OH sites present on the glass surface[46]. Oasis HLB (60 mg, 3 mL, Waters, UK) were used for solid phase extraction (SPE). HQ water, river water (collected in South-West England) and activated sludge (collected from a local wastewater treatment plant) were used for method development and validation.

Synthesis of FL and NFL enantiomers. This procedure employs 3-chloro-1-phenyl-1-propanol as starting material and source of chirality. This was purchased from Sigma-Aldrich chemical company: (*R*)-enantiomer product #338419; (*S*)-enantiomer product #324612. Both were certified as having 99% enantiomeric excess. Experimental procedures are described for the (*R*)-enantiomer; identical procedures were carried out with the (*S*)-enantiomer of starting material to synthesize (*S*)-NFL and (*S*)-FL.

Step 1: (R)-3-phthalimido-1-phenylpropanol (2). At room temperature, to a stirring suspension of potassium phthalimide (3.93 g, 21.25 mmol) in dry dimethylformamide (DMF) (115 mL) was added (*R*)-3-chloro-1-phenyl-1-propanol **1** (3.00 g, 17.65 mmol) in dry DMF (5 mL). The reaction mixture was heated to 90 °C and left to stir for 2 hours, until completion was observed by thin layer chromatography (TLC.) To the cooled reaction mixture was added H_2O (300 mL), and extracted with diethyl ether (2 × 300 mL). The combined organic extracts were washed with a saturated solution of LiCl (300 mL), brine (300 mL), dried over $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to give (*R*)-3-phthalimido-1-phenylpropanol **2** as a white powder (4.30 g, 88%); mp 78–79 °C; R_f 0.36 (3:1 Petrol/ethyl acetate). δ_H (250 MHz, $CDCl_3$) 7.86–7.83 (2 H, m, ArH), 7.74–7.71 (2 H, m, ArH), 7.36–7.21 (5 H, m, ArH), 4.69 (1 H, t, *J* 6.5 Hz, CHOH), 3.91 (2 H, t, *J* 6.5 Hz, CH_2N), 2.13–2.05 (2 H, m, CH_2CHOH); δ_C (300 MHz, $CDCl_3$) 168.8, 143.5, 134.0, 131.9, 128.4, 127.4, 125.6, 123.3, 71.2, 37.6, 34.8 (Figure S3).

Step 2: (R)-3-amino-1-phenyl-1-propanol (3). At room temperature, to a stirred solution of (*R*)-3-phthalimido-1-phenylpropanol **2** (4.10 g, 14.5 mmol) in EtOH (90 mL) was added hydrazine hydrate (2.09 mL, 43.5 mmol). The reaction mixture was stirred for 1 hour and then heated to reflux for 2 hours. The reaction mixture became thick and cloudy upon heating, and when cooled, precipitate was filtered off. Recovered filtrate was concentrated under reduced pressure, diluted with dichloromethane (DCM) (10 mL) and filtered, washed with DCM (2×5 mL). The recovered filtrate was concentrated *in vacuo* to give the title compound (*R*)-3-amino-1-phenyl-1-propanol **3** as a brown oil (1.85 g, 85%); R_f 0.09 (100:10:1 DCM/MeOH/ Et_3N). δ_H (300 MHz, dimethylsulfoxide (DMSO-d_6)) 7.34–7.27 (4 H, m, ArH), 7.24–7.17 (1 H, m, ArH), 4.66 (1 H, dd, *J* 7.0, 6.0 Hz Hz CHOH), 2.71–2.60 (2 H, m, CH_2N), 1.69–1.62 (2 H, m, CH_2CHOH); δ_C (75 MHz, DMSO-d_6) 146.6, 128.0, 126.6, 125.7, 71.3, 42.2, 38.9 (Figure S4).

Step 3: (R)-3-Phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine•HCl [(R)-NFL hydrochloride salt]. At 0 °C, to a stirred suspension of sodium hydride (60% in oil, 0.73 g, 18.34 mmol) in DMSO (3.0 mL) was added (*R*)-3-amino-1-phenyl-1-propanol **3** (1.85 g, 12.23 mmol) in DMSO (1.0 mL). The reaction mixture was stirred at 55 °C for 30 min and 4-fluorobenzotrifluoride (3.01 g, 18.34 mmol) in 1.85 mL DMSO was added dropwise. The resulting mixture was heated for 1 hour at 90 °C, until completion was observed by TLC. The mixture was cooled to 0 °C, and diluted with aqueous 1 N NaOH (20 mL). Toluene was used to extract the product (3×20 mL), and combined organic extracts were dried over $MgSO_4$ and filtered. The crude product was purified by column chromatography (100:0:1 to 100:6:1 DCM/MeOH/Et_3N) to give (*R*)-3-phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine [(*R*)-NFL] as a brown oil (2.10 g, 58%). Product (1.0 g, 3.39 mmol) was dissolved in 4 M HCl in dioxane (10.0 mL, 40 mmol), and left to stir for 2 hours. Reaction mixture was concentrated *in vacuo*, and recrystallized (50 mL of 3:2 diethyl ether/hexane) to give (*R*)-3-Phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine hydrochloride [(*R*)-NFL•HCl] as a white solid (0.80 g, 71%); mp 128–129 °C; R_f 0.04; δ_H (250 MHz, $CDCl_3$) 8.45 (3 H, br s, NH_3) 7.39 (2 H, d, *J* 8.5 Hz, ArH), 7.27–7.32 (5 H, m, ArH), 6.91 (2 H, d, *J* 8.5 Hz, ArH), 5.42 (1 H, dd, *J* 7.5, 4.5 Hz, CHO), 3.18 (2 H, app t, *J* 5.5 Hz, CH_2CH_2NH), 2.47–2.27 (2 H, m, CH_2N); δ_C (300 MHz, $CDCl_3$): 159.5, 139.0, 129.1, 128.4, 126.7 (q, $^3J_{CF}$ 3.8 Hz), 125.7, 124.2 (q, $^1J_{CF}$ 270 Hz), 123.3 (q, $^2J_{CF}$ 32.6 Hz), 115.9, 77.4, 36.9, 36.0; ν_{max} (film) 3385 (N-H), 2891 (C-H), 2015, 1613 cm^{-1}; $[\alpha]_D + 14.0°$ (*c* 1, $CHCl_3$); for (*S*)-enantiomer: $[\alpha]_D$ −15.0° (*c* 1, $CHCl_3$) (Figure S5).

Step 4: (R)-N-Methyl-3-(4-trifluoromethylphenoxy)-3-phenylpropylamine [(R)-FL]. At room temperature, to a stirred solution of 3-phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine (*R*)-NFL (1.0 g, 3.38 mmol) and methyl chloroformate (0.29 mL, 3.72 mmol) in DCM (15.0 mL) was added aqueous K_2CO_3 (2.33 g, 16.89 mmol in 30 mL H_2O). The reaction mixture was vigorously stirred for 20 minutes, until completion was observed by TLC, dyed with ninhydrin. The organic phase was separated and the aqueous phase extracted with DCM (2×30 mL). The combined organic extracts were dried over $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to yield intermediate carbamate as a pale yellow oil. At 0 °C, to a stirring suspension of $LiAlH_4$ (0.25 g, 6.59 mmol) in dry tetrahydrofuran (THF) (15.0 mL) was added dropwise a solution of the intermediate carbamate in dry THF (5.0 mL). The reaction mixture was gradually heated to reflux for 2 hours. To the cooled mixture were cautiously added 0.25 mL of water, followed by 0.25 mL of 2 N NaOH, and 0.75 mL of water, in that order. The solution was dried over $MgSO_4$ and filtered. The filtrate was concentrated over reduced pressure, and the crude product was purified by column chromatography (100:0:1 to 100:1:1 EtOAc/MeOH/Et_3N) to give (*R*)-*N*-Methyl-3-(4-trifluoromethylphenoxy)-3-phenylpropylamine [(*R*)-FL] as a pale yellow oil (0.84 g, 80%); R_f 0.09 (100:1:1 EtOAc/MeOH/Et_3N); δ_H (300 MHz, $CDCl_3$) 7.43 (2 H, d, *J* 8.5 Hz, ArH), 7.34–7.23 (5 H, m, ArH), 6.90 (2 H, d, *J* 8.5 Hz, ArH), 5.31 (1 H, dd, *J* 8.0, 4.5 Hz, CHO), 2.82–2.66 (2 H, m, CH_2CH_2NH), 2.44 (3 H, s, CH_3), 2.27–2.16 (1 H, m, CHHN), 2.08–1.97 (1 H, m, CHHN), 1.69 (1 H, br. s, NH); δ_C (300 MHz, $CDCl_3$) 160.5, 141.0, 128.8, 127.8, 126.7 (q, $^3J_{CF}$ 3.8 Hz), 125.7, 115.7, 78.5, 48.2, 38.6, 36.4 (signals for -CF_3 and C-CF_3 were not observed); ν_{max} (film) 3033 (ArC-H), 2937 (ArC-H), 2846 (C-H), 2796, 1614 cm^{-1}; $[\alpha]_D + 3.0°$ (*c* 1, $CHCl_3$); for (*S*)-enantiomer: $[\alpha]_D$ – 3.0° (*c* 1, $CHCl_3$) (Figure S6).

Microcosm bioreactors. *River water simulating microcosms.* River water microcosm experiments were conducted in light (L) and dark (D) conditions, and biotic (B) or abiotic (A) conditions with or without sodium azide (an inhibitor of microbial processes) respectively as shown in Fig. 6. Four microcosm bioreactors were investigated in duplicate thus eight autoclaved conical flasks were used as bioreactors in microcosm experiments. Each bioreactor was filled with 2 L of river water collected from a local river and spiked with racemic standard of *S*/*R* (±) FL to obtain a final concentration of 1 µg L^{-1}. Abiotic bioreactors were spiked with sodium azide at a concentration of 1 g L^{-1} to inhibit biotic processes. Two replicates of biotic and abiotic bioreactors were exposed to light and another two replicates of each bioreactor were kept in the dark. Light conditions were simulated with an Osram 400 W HQI BT daylight lamp during 8 h each day. Average photon flux measured at the level of the bottle base was 395 µmol m^{-2} s^{-1}. Dark conditions were simulated covering up the flask with foil. Magnetic stirrers were used to ensure good mixing. The experiment was carried out during 16 days. Samples (50 mL each) were collected every day, with the exception of weekends. IS was added to each sample (to obtain a final concentration of 100 ng mL^{-1}). Samples were then frozen to prevent compound degradation until their analysis.

Dissolved oxygen (DO), pH and temperature (T) were analyzed during sampling period and total suspended solids (TSS), NO_2^-, NH_4, and chemical oxygen demand (COD) were analyzed at the beginning of the experimental period (Table S16).

Activated sludge simulating microcosms. Activated sludge microcosm experiments were conducted in the dark and aerobic conditions. Three microcosm bioreactors were investigated in duplicate. They were filled with 2 L of

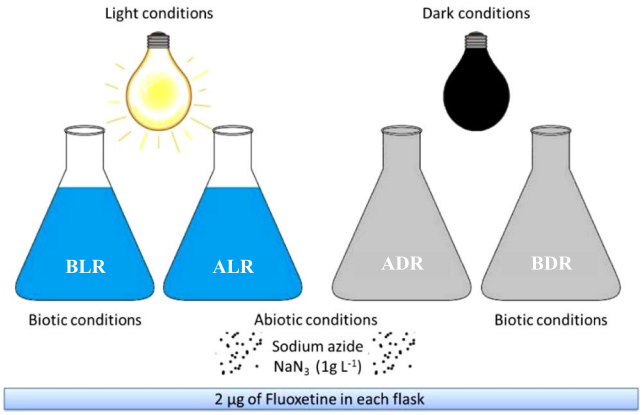

Figure 6. Scheme of river and activated sludge simulating microcosms.

fresh activated sludge collected from a local wastewater treatment plant. One bioreactor remained un-spiked and the other two bioreactors were spiked with a racemic standard of *S*/*R* (±) FL to obtain concentrations of either 10 or 100 µg L^{-1}. Dark conditions were simulated by covering up the flask with foil. Aerobic conditions were obtained using air from BOC air cylinder and a thorough mixing was maintained using magnetic stirrers. The experiment was 24 hours long. Samples (100 mL each) were taken at the following time intervals: 0, 0.5, 1, 1.5, 2, 3, 5, 8, 12 and 24 h. After sample collection, IS was added to each sample to obtain a final concentration of 100 ng mL^{-1}. Collected samples were subsequently frozen until their analysis to prevent degradation of compounds.

DO, pH, T and TOC, were analyzed throughout the experimental period and TSS, NO_3^-, NO_2^-, NH_4 and COD were analyzed at the beginning of the experiment at t = 0 min (Table S17).

Kinetics – activated sludge simulating microcosms. The compounds studied are characterized as having low volatility and therefore volatilization was not considered as a potential removal pathway in studied microcosms. Photodegradation was also not considered (not relevant) under tested activated sludge conditions. Therefore the two important degradation mechanisms to consider were biodegradation and sorption to sludge. FL was reported to have high sorption affinity towards particulate matter[47]. However, in this study, sorption equilibrium was assumed and therefore sorption could be considered negligible. This is because sorption is assumed to be fast when compared to biological degradation[48]. Section "*Activated sludge microcosm*" confirms this hypothesis as FL in activated sludge microcosms remained constant in FL 100 µg L^{-1} microcosm during the first 2 h of the experiment. In the case of FL 10 µg L^{-1}, a significant drop of FL concentration took place during the first 0.5 h (likely due to sorption) and then remained stable during the first 3 h of the experiment, showing a significant lag phase.

Several reports utilized pseudo-first-order kinetics for degradation of micropollutants in activated sludge reactors[48–50]. Indeed when applying pseudo-first order kinetics (OECD 303) in this work, $\ln(C_e/C_i)$ plotted as a function of time yielded a straight line ($R^2 > 0.9$). Pseudo-first order biodegradation rate k_1 [L $g^{-1}h^{-1}$] (normalised for concentration of suspended solids) was therefore calculated using the following formula (equation (1)):

$$In\ \frac{C_e}{C_i} = -k_1 * t * SS \tag{1}$$

where: t = aeration time (24 h), C_e = concentration at time point t (µg L^{-1}), C_i = initial concentration (µg L^{-1}), SS = concentration of activated sludge solids (g L^{-1}).

Analysis. *Solid phase extraction.* Samples (50 mL of river water and 100 mL of activated sludge) were filtered using Whatman GF/F 0.7 µm glass fiber filter and passed through Oasis HLB cartridges (60 mg, 3 mL) pre-conditioned with 2 mL of MeOH and equilibrated with 2 mL of HQ water at a rate of 8 mL min^{-1}. Samples were passed through the HLB cartridge at a rate of 8 mL min^{-1} and then dried under the vacuum for 30 min to dry out residual water. Analytes were eluted with 4 mL of MeOH at a rate of <1 mL min^{-1}. Extracts were then evaporated to dryness with TurboVap evaporator (Caliper, UK, 40 °C, N_2, <5 psi) and reconstituted in 0.5 mL of mobile phase. All samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13 mm) and transferred to popylpropylene 0.3 mL capacity vials (Waters, UK).

SPE recoveries of FL and NFL in HQ water, river water and activated sludge were calculated as the ratio of the analyte peak area in the sample extract spiked with analytes before extraction (the peak area of analyte un-spiked sample extract was subtracted) to the analyte peak area in the non-extracted standard solution.

ME was calculated for each chiral drug as a percentage decrease or increase in signal intensity in a sample matrix versus HQ water using the following equation (2):

$$ME = \frac{\Delta matrix}{\Delta HQ\ water} \tag{2}$$

where Δmatrix is the standard calibration graph slope in different matrix (river water or active sludge) and ΔHQ water is the standard calibration graph slope in HQ water.

Chiral-LC-MS/MS method. Chromatographic analysis was performed using an Acquity UPLC system (Waters, Manchester, UK) consisting of Acquity UPLC binary solvent manager and Acquity UPLC sample manager. To achieve suitable separation of FL and NFL and their two stereoisomers, two chiral columns, namely Chiralpak CBH (10 cm × 2.0 mm, 5 μm particle size) and Astec CBV (25 cm × 2.1 mm, 5 μm particle size) were screened.

Several mobile phases were tested in order to obtain chiral separation of FL and NFL using LC and to maintain satisfactory electrospray ionization (ESI) performance in the positive ionization mode. MeOH, EtOH and HQ water were used at different concentrations as the mobile phase solvents. Among the mobile phase additives, different concentrations of AAC (1, 4 and 10 mM), MeOH (85, 80 and 70%) and EtOH (70 and 80%) were tested to maximize the R_s of FL and NFL.

The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. R_s was calculated using the following equation (3):

$$R_S = \frac{2(tr_{E2} - tr_{E1})}{W_{E2} + W_{E1}} \tag{3}$$

where tr_{E1} and tr_{E2} are the retention times of the first and the second eluted enantiomers, respectively, and W_{E1} and W_{E2} are the widths of these responses at the base peak.

The best enantiomeric separation of the studied drugs was achieved with a mobile phase (pH 6.5) composed of 70% of EtOH, 30% HQ water, 4 mM of AAC and 0.005% of FA using an Astec CBV column. The separation of enantiomers of chiral pharmaceuticals was undertaken under isocratic conditions, with an injection volume of 20 μL. The column was kept at 25 °C and the temperature in the sample manager was kept at 4 °C. The flow rate was 0.06 mL min^{-1}, which gave an initial pressure of ~850 psi.

Identification and quantification of FL and NFL was undertaken with an Acquity Xevo TQD (Waters, Manchester, UK), a triple quadrupole MS equipped with an ESI source. The analyses were performed in positive mode with a capillary voltage of 3 kV, a source temperature of 150 °C and a desolvation temperature of 250 °C. A cone gas flow of 50 L h^{-1} and desolvation gas flow of 450 L h^{-1} were used. Nitrogen, used as a nebulising and desolvation gas, was provided by a high purity nitrogen generator (Peak Scientific Instruments Ltd., UK). Argon (99.99%) was used as a collision gas. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, measuring the fragmentation of the protonated pseudo-molecular ions of each compound. A dwell time of 20 ms per ion pair was optimized to maintain high sensitivity of the analysis. MassLynx v4.1 (Waters, UK) software was used to collect and analyze the obtained data.

Quantifier and qualifier transitions were optimized for each compound based on the most intense signal. Specific parameters such as collision energy (CE) and cone voltage (CV) were optimized for FL, NFL and FL-d5 separately in a continuous flow mode through direct injection of standard solution at a concentration of 50 μg L^{-1} into the stream of the mobile phase. FL presents a precursor ion $[M + H]^+$ m/z of 310.3 and a product ion of m/z 44.2 (quantifier transition) with a CV of 34 and CE of 10 and m/z 148.2 (qualifier transition) with a CV of 25 and CE of 8. NFL presents a precursor ion $[M + H]^+$ m/z 298.4 and a corresponding product ions at m/z 134.1 (quantifier transition) with a CV of 17 and CE of 7 and m/z 30 (qualifier transition) with a CV of 17 and CE of 7. FL-d5 presents a precursor ion $[M - H]^+$ m/z 315.2 and a corresponding product ions at m/z 136.2 (quantifier transition) with a CV of 26 and CE of 71 and m/z 20.

Method validation parameters. A 10-point multi-component IS calibration curve was applied for quantification of FL and NFL enantiomers. All instrumental and method validation parameters such as linearity and range, accuracy, precision, detection and quantification limits and calibration curve were determined for HQ water, river water and activated sludge spiked with known concentrations of chiral compounds.

Linearity and range of the analytical procedure were undertaken by serial dilution of stock solution (10 μg mL^{-1}). Accuracy of the method was evaluated at three concentration levels (5, 25 and 250 μg L^{-1}) as a percentage deviation from known added quantity of each enantiomer in the sample. Intra-day and inter-day precision was expressed by the relative standard deviation (RSD) of 3 replicate measurements at three different concentration levels (5, 25 and 250 μg L^{-1}) on the same and three different days.

HQ water standard solutions were used for instrumental detection limit (IDL) and instrumental quantification limit (IQL). The IQL was estimated for the concentration of a compound that gave a signal-to-noise ratio of 10:1. The IDL corresponded to the concentration that gave a signal-to-noise of 3:1.

MDL and (MQL for river water and activated sludge were calculated using the following equations (4) and (5):

$$MDL = \frac{IDL_{S/N} \times 100}{Rec \times CF} \tag{4}$$

$$MQLcalc = \frac{IDL_{S/N} \times 100}{Rec \times CF} \tag{5}$$

where $IDL_{S/N}$ is the instrumental detection limit (μg L^{-1}), $IQL_{S/N}$ is the instrumental quantification limit (μg L^{-1}), Rec is the absolute recovery of the analyte (%) at 25 μg L^{-1}, and CF is the concentration factor, which in this method denotes 200 for active sludge and 100 for river water.

The EF of studied chiral drugs was calculated using the following equation (6):

$$EF = \frac{(S)}{(S) + (R)} \quad (6)$$

Where (*S*) and (*R*) are concentrations of the (*S*)- and (*R*)- enantiomers, respectively.

Toxicity tests. *Daphnia magna acute 48 h immobilization assay.* The *D. magna* bioassay was carried out using Daphtoxkit™ (Crustacean Toxicity Screening Test for Freshwater; Microbiotests, Nazareth, Belgium) following the standard operational procedure in accordance to the ISO standard 6341:2012 and the OECD 202 guideline. Less than 24 h old daphnids were exposed to a series of concentrations of each enantiomer of FL and NFL. Six concentration levels (5 concentrations plus control, four replicate beakers for each concentrations, five individual for each beaker) were tested. The concentrations were from 0.5 to 50 mg L^{-1} for FL and NFL enantiomers based on preliminary range finding tests. Each experiment was repeated in triplicate. After 48 h incubation, daphnids were observed and the mobile daphnids in each container were reported. The EC50 were calculated using 48 h results.

Tetrahymena thermophila chronic 24 h population growth assay. *T. thermophila* bioassay was carried out using Protoxkit F (Microbiotests, Ghent University, Belgium). The tests were performed in accordance with the protocols provided by the manufacturer. Protoxkit F is a 24 h chronic population growth assay.

The tests were carried out in disposable spectrophotometric cells of 1 cm path-length, to enable the measurement of the optical density (OD) at 440 nm. These measurements were taken at T0h and T24h, as well as two hour increments after initial 24 h incubation to monitor the change in turbidity of the sample. The reconstituted food substrate supplied with each test provides an initial high turbidity at T0h, which, in the control cells, drastically decreases over the next 24 h due to the uninhibited growth of the ciliate population. This change in OD over the time period is used to quantify the degree of inhibition and subsequent calculation of the EC50. Each concentration was repeated in duplicate.

The initial protozoa inoculum was prepared by measuring an aliquot of the live suspension using photometry absorbance at 440 nm and diluted to achieve a theoretical OD value of 0.040. Each test cell is inoculated with 40 µL of this suspension, lending to an approximate population density of 100 protozoa per milliliter.

An initial study was carried out to find the approximate range of uninhibited growth and 100% inhibition for each enantiomer/compound across 7 orders of magnitude. The definitive toxicity test was carried out from the lowest concentration with a percentage population growth inhibition of 80–100% to the highest concentration with an inhibition between 0–20%. To ensure the test was valid the control must reach 60% OD decrease after 24 h. In some tests this may take 2–4 h longer than 24 h, this is batch dependent and indicates a slightly slower growth of the ciliates, however this is still considered valid. In this case all the tests carried out were with the same batch which took 28 h to fulfill the validation criteria. The EC50 values were calculated using 28 h results.

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Author Contributions

M.J.A.C., K.P. and B.K.H. planned and designed the study. M.J.A.C. undertook microcosm experiments. KP undertook toxicity experiments. M.T.S., A.P.G.. and SEL synthesised enantiomers of fluoxetine and norfluxetine. The manuscript was drafted by MJAC and BKH with contributions from all co-authors. All authors are aware of the content and accept responsibility for the manuscript.

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Appendix 6B: Statement of Authorship

This declaration concerns the article entitled:			
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Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [X]			
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Candidate's contribution to the paper (provide details, and also indicate as a percentage)	Formulation of ideas: The candidate was the minor contributor in the formulation of the original idea. Design of methodology: The candidate was a minor contributor to the design of the study Experimental work: The candidate was the major contributor in the use of *Tetrahymena thermophila* ecotoxicity test. Presentation of data in journal format: The candidate was the major contributor for the data analysis, interpretation, and presentation of the data of the *T. thermophila* test and assisted in the writing of the paper.		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed	[redacted]	**Date**	11/12/2020

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Stereochemistry of ephedrine and its environmental significance: exposure and effects directed approach

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Stereochemistry of ephedrine and its environmental significance: Exposure and effects directed approach

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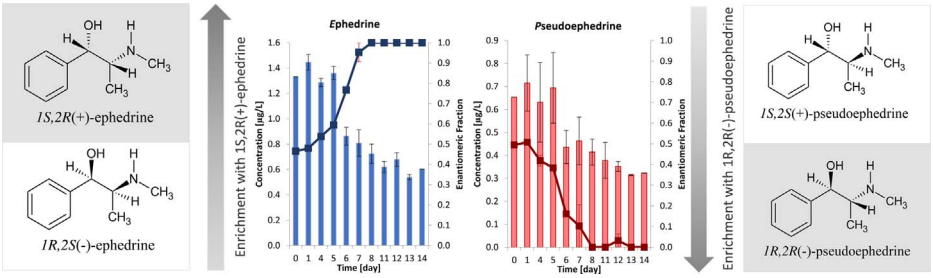
GRAPHICAL ABSTRACT



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ABSTRACT

Analysis of drugs and pharmaceuticals in the environment is typically performed with non-chiral chromatographic techniques. The environmental risks posed by chiral compounds analysed in this way must therefore be assumed to be independent of chirality, meaning that each enantiomer is equally potent in toxicity and long-lived in stability. This manuscript examines the degradation of each of the four isomers of ephedrine in river simulating microcosms and links this to toxicity data obtained by exposing three different organisms (*D. magna, P. subcapitata and T. thermophila*) to each of the isomers individually. Microcosms showed that significant degradation only occurred in biotic conditions and that only two isomers (*1R,2S*-(-)-ephedrine, *1S,2S*-(+)-pseudoephedrine) degraded significantly over a period of fourteen days. This is concerning because at least one of the non-degraded isomers (*1S,2R*-(+)-ephedrine) has been observed in wastewater effluent, which discharges directly into rivers, meaning these isomers could be persistent in the environment. We also observed formation of *1S,2R*-(+)-ephedrine in single isomer *1R,2S*-(-)-ephedrine river simulating microcosms. Human liver microsome assays and mass spectrometry based data mining revealed that *1S,2R*-(+)-ephedrine is not human derived but it could be formed as a results of microbial metabolic processes. Across all three organisms tested the persistent isomers (*1S,2R*-(+)-ephedrine and *1R,2R*-(-)-pseudoephedrine) were more toxic than those that undergo degradation; meaning that if these isomers are entering or formed in the environment they might represent a potentially hazardous contaminant.

1. Introduction

Ephedrine has two chiral centres and can therefore exist in the form of four stereoisomers: *1R,2S*-(-)-ephedrine, *1S,2R*-(+)-ephedrine, *1S,2S*-(+)-pseudoephedrine and *1R,2R*-(-)-pseudoephedrine (Table 1). However, only two stereoisomers: *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine are believed to exist in natural sources such as *ephedra*. *1R,2S*-(-)-ephedrine

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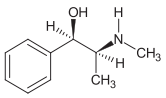
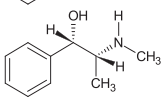
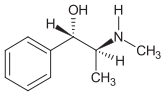
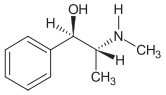
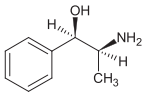
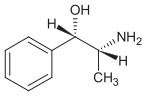
Table 1
Studied chemicals and their properties.

Compound	Structure	CAS No
1R,2S-(-)-ephedrine		299-42-3
1S,2R-(+)-ephedrine		24221-86-1
1S,2S-(+)-pseudoephedrine		345-78-8
1R,2R-(-)-pseudoephedrine		321-97-1
1R,2S-(-)-norephedrine		492-41-1
1S,2R-(+)-norephedrine		37577-28-9

finds wide applications as a bronchodilator to treat bronchospasm associated with asthma, bronchitis and emphysema. It is also abused for its stimulant properties. *1S,2S*-(+)-pseudoephedrine is used as a decongestant [1].

Ephedrine has been detected previously in environmental matrices [2,3] but with the usage of non-enantioselective methodology, which did not allow for stereoisomeric profiling of ephedrine. As a result this did not allow for an accurate assessment of the possible effects ephedrine might have on the environment. Stereoisomeric profiling is of vital importance as different stereoisomers of ephedrine differ significantly in potency, *1R,2S*-(-)-ephedrine has much higher stimulant properties than *1S,2S*-(+)-pseudoephedrine, and possibly also in toxicity to certain organisms.

In our previous study [1], a verification of the enantiomer-specific fate of ephedrine isomers was undertaken in a full scale WWTP and in receiving waters. Of the two enantiomers of (±)-ephedrine only the natural *1R,2S*-(-)-ephedrine enantiomer was frequently detected. However, 'non-natural' *1S,2R*-(+)-ephedrine was detected at low levels in only certain WWTPs throughout the sampling campaign, mainly in treated wastewater, which might suggest stereoselective processes occurring during treatment (e.g. chiral inversion although there is currently no experimental evidence to support this claim) leading to enrichment of ephedrine with *1S,2R*-(+)-enantiomer. It is worth noting that the most prevalent formation of *1S,2R*-(+)-ephedrine occurred during spring/summer time due to potentially higher activity of microorganisms. The possibility of chiral inversion occurring during treatment is of critical importance in understanding the fate of ephedrines in the environment and has to be studied further.

The verification of cumulative concentrations of ephedrines in raw wastewater indicated that higher levels of these compounds were observed during winter time (reaching 180 g/day in February in all studied WWTPs) than during summer time (< 80 g/day in August) [1,4]. Interestingly, the analysis of diastereomeric fractions (DFs) of natural *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine in raw wastewater revealed that over the winter months ephedrines were enriched with *1S,2S*-(+)-pseudoephedrine. This is possibly due to higher usage of over-the-counter medications (containing *1S,2S*-(+)-pseudoephedrine) for the treatment of mild symptoms of cold. During the spring and summer months a reverse situation was observed as ephedrine was found to be enriched with a much more potent stimulant, *1R,2S*-(-)-ephedrine. This is a very important finding indicating that non-enantioselective measurement of ephedrines cannot be a reliable indicator of actual potency of ephedrines in the environment. Higher cumulative concentrations of ephedrines, which are enriched with less potent *1S,2S*-(+)-pseudoephedrine (as was observed during winter time in this study) might be of lower environmental significance than lower concentrations of ephedrines enriched with much more potent *1R,2S*-(-)-ephedrine (in summer in this study). Furthermore, wastewater treatment resulted in almost all cases in further enrichment of ephedrines in the aqueous phase with more potent *1R,2S*-(-)-ephedrine, with an average increase in DFs from 0.25 in raw wastewater to 0.35 in treated wastewater. Interestingly, the monitoring of receiving waters revealed that ephedrine was enriched with *1R,2S*-(-)-ephedrine at the beginning of the course of the river and its DFs decreased over the course of the river indicating an increase of *1S,2S*-(+)-pseudoephedrine (e.g. during the August sampling campaign DF of ephedrine denoted 0.93 ± 0.03 at the beginning of the river course and decreased to 0.33 ± 0.03 over 50 km downstream); a reverse situation to the one observed during wastewater treatment. This might indicate that different microbial communities are responsible for transformation of ephedrines during wastewater treatment and in the environment.

The absence of 1S,2R-(+)-ephedrine in influent wastewater suggests that it is not formed by metabolism of ephedrine in humans. However previous research into human metabolism of ephedrine was not carried out in a stereoselective manner and is mostly limited to 1R,2S-(-)-ephedrine, rather than the more prescribed and environmentally abundant 1S,2S-(+)-pseudoephedrine. Metabolic data indicates that 1R,2S-(-)-ephedrine is excreted primarily unchanged, with norephedrine and other metabolites forming in smaller quantities [5,6]. Whilst literature data was not available for the metabolism of other ephedrine isomers in humans, metabolism of 1S,2R-(+)-ephedrine in rabbits and rabbit liver microsomes was observed to be slower than metabolism of 1R,2S-(-)-ephedrine [7]. Whilst this shows that a metabolic preference for the naturally occurring isomer, which may be important for the formation of synthetic isomers in the environment, rabbit metabolism of ephedrine is different from human metabolism with only a small percentage of (±)-ephedrine excreted [7,8] or isolated from rabbit liver microsomes [7].

Our previous research raises several questions undermining validity of widely accepted environmental risk assessment procedures for pharmacologically active compounds. This includes lack of appreciation of the phenomenon of chirality in environmental risk assessment (ERA) for human and veterinary medicines [9,10]. This paper attempts to answer the most urgent questions regarding the significance of stereochemistry of pharmaceuticals (using stereoisomers of ephedrine as a model example) in the context of their environmental fate and effects. To the authors' knowledge this is the first report tackling stereoselective transformation of ephedrines in river simulating microcosms and associated enantiomer-specific ecotoxicity.

2. Experimental

2.1. Chemicals and materials

Reference standards: *1R,2S*-(-)-ephedrine [(-)-Eph], *1S,2R*-(+)-ephedrine [(+)-Eph], *1S,2S*-(+)-pseudoephedrine [(+)-Pse], *1R,2R*-(-)-pseudoephedrine [(-)-Pse], *1R,2S*-(-)-norephedrine [(-)-NorEph] and *1S,2R*-(+)-norephedrine [(+)-NorEph] were of ≥98% purity and were purchased from Sigma-Aldrich (Gillingham, UK). Surrogate/internal standards (SS/IS): 1S,2R-(+)-ephedrine-d3 (CAS No. 285979-73-9) and *R/S*-(±)-methamphetamine-d5 (CAS No. 60124-88-1, were purchased from Sigma Aldrich (Gillingham, UK) and Toronto Research Chemicals (Canada) respectively (Table 1). All surrogate/internal standards were added to the samples before extraction and were also used for the quantification of the analytes.

2.2. Sample preparation and analysis

Chiral drugs were extracted from surface water (50 mL) using Solid-Phase Extraction (SPE) and Oasis HLB cartridges (Waters, UK). All samples were spiked with 50 ng of each surrogate/internal standard and filtered with GF/F filters. Analytes were then eluted with 4 mL of MeOH and the extracts evaporated to dryness under nitrogen with a TurboVap evaporator (Caliper, UK, 40 °C, N_2, 5–15 psi) and reconstituted in 0.5 mL of mobile phase.

Waters ACQUITY UPLC™ system (Waters, Manchester, UK) equipped with Chiral-CBH column, 100 × 2 mm, 5 µm (Chromtech, Congleton, UK) and Chiral-CBH 10 × 2.0 mm guard column (Chromtech, Congleton, UK) were used for the analysis of enantiomers of ephedrines. The separation of ephedrines was undertaken using two different methods depending on the experiment. Samples from river simulating microcosm experiments were analysed using the method described by Evans et al. [11]. The elution order of the four ephedrine isomers was: 1S,2R-(+)-ephedrine, 1R,2R-(-)-pseudoephedrine, 1R,2S-(-)-ephedrine, 1S,2S-(+)-pseudoephedrine. Samples from human liver microsome experiments were analysed using the method described by Castrignanò et al. [12]. The elution order of the four ephedrine isomers was the same as in the previous method, but with co-elution of 1R,2S-(-)-ephedrine and 1R,2R-(-)-pseudoephedrine. This co-elution was not considered a hindrance for the analysis undertaken in the human liver microsome experiments. An injection volume of 20 µL was used in both experiments. Method validation parameters for both methods are summarised in Tables S1–4.

A XevoTQD (triple quadrupole) mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionisation source (ESI) was used for the quantification of ephedrines in both methods. The analyses were performed in ESI positive mode using multiple-reaction monitoring (MRM). Nitrogen was used as the nebulising gas at a flow rate of 500 L/Hr, supplied by a high-purity nitrogen generator (Waters, Manchester, UK). Argon (99.998%) was used as the collision gas and supplied by BOC cylinder. MassLynx 4.1 (Waters, UK) and TargetLynx (Waters, UK) software was used to collect and analyse the obtained data.

The relative concentration of enantiomers of chiral drugs was expressed as the enantiomeric fraction (*EF*) and was calculated with the following equation:

$$EF = \frac{C_{(+)-enantiomer}}{C_{(+)-enantiomer} + C_{(-)-enantiomer}} \quad (1)$$

Where C-*(+)-enantiomer* and C-*(-)-enantiomer* are concentrations for the (+) and (-) enantiomers of (±)-ephedrine or (±)-pseudoephedrine. *EF* equals 1 or 0 in the case of single enantiomer form and 0.5 in the case of racemate.

A range of common inorganic ions (NO_2^-, NO_3^- and NH_4^+) and chemical oxygen demand (COD) were quantified to assess environmental conditions at the time of sampling and for experimental monitoring. Commercially available testing kits (purchased from Merck) were used and concentrations determined photochemically utilising a Merck Spectroquant® Pharo 300 spectrophotometer. Collected surface water was tested before spiking with ephedrine and again after a two week testing period.

2.3. River simulating microcosm experiments

2.3.1. Mixed river simulating microcosms

Mixed compound microcosm experiments were conducted to investigate the fate of ephedrines at an enantiomeric level due to biodegradation, photodegradation and other abiotic processes including sorption. River water for the microcosm bioreactor experiments was collected from a large river in the South-West of the UK in November.

Degradation experiments were conducted with and without light, to study photochemical and physical processes, e.g. hydrolysis and sorption, and with or without sodium azide, as an inhibitor to biotic processes (see Fig. S1). Eight conical flasks, made of borosilicate 3.3 glass with no visible light absorption and UV light cut-off at < 275 nm, were used as bioreactors in all microcosm experiments and were autoclaved prior to use. All were subsequently spiked with 1 µg/L each of 1S,2R-(+)-ephedrine, 1R,2S-(-)-ephedrine, 1S,2S-(+)-pseudoephedrine, 1R,2R-(-)-pseudoephedrine, 1R,2S-(-)-norephedrine and 1S,2R-(+)-norephedrine. Norephedrine was tested alongside ephedrines as it is the primary metabolite of ephedrine [5]. Microcosms were then filled with 2 L of unfiltered river water and four were spiked with sodium azide to a concentration of 1 g/L to inhibit biotic processes (Abiotic Reactors). Four bioreactors remained un-spiked in order to allow biotic processes to occur (Biotic Reactors). Two biotic and two abiotic reactors were then wrapped in aluminium foil (Dark Reactors) and the remaining two biotic and two abiotic reactors left uncovered (Light Reactors). To limit outside contamination of the microcosms, all eight were plugged at the top with cotton wool, as this still allowed for the flow of air into the microcosm. Finally, each reactor had a magnetic stirrer bar added and were placed onto unheated magnetic stirring pads at the lowest speed that allowed a vortex to form.

Daylight conditions were simulated using an Osram400W powerstar® HGI®-BT daylight lamp, which was switched on for eight hours each day to mimic average sunlight conditions in the UK. The bulb provides 23.64 µmol S^{-1} m^{-2} per microamp of illumination at the 2 L mark of a 2 L conical flask, with the probe facing perpendicular to light source. The bulb provides 158.34 µmol S^{-1} m^{-2} per microamp of illumination at the bottom of a dry 2 L conical flask, with the probe directly facing the light source. All light intensity measurements were made using a LI-250A light meter with a quantum sensor. In order to decrease the effect of heat generated by the lamp all the microcosms, including those in the dark, were cooled using fans to ensure a roughly equal temperature inside each microcosm.

Samples were taken at regular intervals (once per day) over a fifteen-day sampling period and analysed as detailed by Evans et al. [11]. Other parameters analysed during the sampling period included dissolved oxygen (DO), pH, temperature, COD, ammonium, nitrate and nitrite (Fig. S2).

2.3.2. Single-isomer ephedrine river simulating microcosms

Single-isomer ephedrine microcosms were carried out as an extension of the river water simulating microcosms described in 2.3.1, to examine the effects of chirality on ephedrine degradation. The microcosms were set up as described previously using river water collected from a large river in the South West of the UK in February and spiked with 1 µg/L of either 1R,2S-(-)-ephedrine or 1S,2S-(+)-pseudoephedrine, the naturally occurring isomers. Eight microcosms were prepared in total (see Fig. S3): four containing 1R,2S-(-)-ephedrine and four containing 1S,2S-(+)-pseudoephedrine. For each set of four single-isomer ephedrine microcosms, two were wrapped in foil (Dark Reactors) and the others left exposed to eight hours a day of simulated daylight (Light Reactors) from an Osram400W powerstar® HGI®-BT daylight lamp as described previously.

Samples were taken at regular intervals (once per day) over a fifteen-day sampling period and analysed as detailed by Evans et al. [11]. Other parameters analysed during the sampling period included dissolved oxygen (DO), pH, temperature, COD, ammonium, nitrate and nitrite (Fig. S4).

2.4. Human liver microsome metabolism

Human liver microsomes (HLMs) were set-up in accordance to the method described by Lopardo et al. [13] and were performed in duplicate for both 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine, with each isomer examined in isolation. Metabolism studies were carried out to investigate if human metabolism of either ephedrine isomer

lead to the formation of any other isomer. Currently available data on (±)-ephedrine metabolism in humans suggests that if metabolised ephedrine will primarily undergo hydroxylation or demethylation [5,6]. The microsomes were incubated for a total of six hours as this matched the expected *in vitro* half-life of 1R,2S-(-)-ephedrine [5]. To determine absolute ephedrine and norephedrine isomer concentrations the samples were analysed as described by Castrignanò et al. [12].

2.5. Retrospective analysis with UHPLC-QTOF – screening for precursors of 1S,2R-(+)-ephedrine

River water samples collected during seven consecutive days in South-West England were analysed in accordance to the method described by Lopardo et al. [13]. Briefly, river water samples were filtered using GF/F glass microfibre filter 0.75 µm (Fisher Scientific, UK) followed by a solid phase extraction (SPE) using HLB Oasis® cartridges Water, UK) and concentrated 400-fold. Extracts were then dried under nitrogen using a TurboVap evaporator (Caliper, UK, 40 °C). Dry extract was then reconstituted in 250 µL 80:20 H_2O:MeOH, transferred to polypropylene vials.

A Dionex Ultimate 3000 HPLC (Thermo Fisher UK Ltd.) coupled with a Bruker Maxis HD Q-TOF (Bruker) equipped with an ESI was utilized for the analysis of extracts. ESI positive and negative mode acquisition was performed in broadband CID acquisition mode. HyStar™ Bruker was used to coordinate the LC–MS system. Chromatographic separation and MS source conditions are described by Lopardo et al. [13].

After analysis, data extracted from the Bruker system were processed with MetID software (Advanced Chemistry Development, Inc., ACD/Labs, UK) in order to predict metabolite structures.

2.6. Toxicity testing

2.6.1. Daphnia magna toxicity tests

The experiment was performed using Daphtoxkit F Magna (Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium) in accordance with test procedures described by national and international organizations (OECD test no. 202 [14]).

24 h–48 h EC50 (or LC50) bioassays were performed in multiwell test plates starting from neonates, uniform in size and in age, hatched from ephippia. In order to provide the neonates hatched from the ephippia with food prior to the test, a 2 h "pre-feeding" was applied with a suspension of *Spirulina* micro-algae. Each ephedrine isomer was tested individually at the following concentrations 7.8, 15.6, 31.3, 62.4, 125, 250, 500, 1000 mg/L. These were prepared by serial dilution of an initial 1 g/L ephedrines solution with standard fresh water [14]. For a statistically acceptable evaluation of the effects each test concentration, as well as the control, were assayed in four replicates of five neonates. *Daphnia magna* neonates were incubated at 20–22 °C for 48 h and the number of dead or immobilised organisms was counted after 24 and 48 h. The EC50/LC50 is the concentration where 50% of the *D. magna* are dead or immobilised, determined by if they can swim freely after gently agitating the solution.

2.6.2. Pseudokirchneriella subcapitata toxicity tests

The experiment was performed using Algaltoxkit F (Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium) in accordance with test procedures prescribed by national and international organizations (e.g. 'Algal growth inhibition test' (OECD Guideline 201 [15]) and the ISO "Water Quality - Freshwater Algal Growth Inhibition Tests with Unicellular Green Algae" (ISO Standard 8692). A 72 h algal growth inhibition test was performed with *Pseudokirchneriella subcapitata* de-immobilized from algal beads. The test is based on the measurement of the optical density of algal cell suspensions (at 670 nm) in spectrophotometric cells of 10 cm path-length. Optical densities were then converted into algal numbers with the aid of the regression formula. The algae density was measured by photometry absorbance at 670 nm and it was diluted with culturing medium to achieve a density of 1.10^6. Each ephedrine isomer was individually diluted to a concentration of 500 mg L^{-1}, 300 mg L^{-1}, 160 mg L^{-1}, 50 mg L^{-1}, 5 mg L^{-1} and 0 mg L^{-1} with culturing medium. Algae were added to these dilutions to achieve a density of 1.10^4 mL^{-1}. 25 mL of this solution was placed in 10 cm spectrophotometer cells, in triplicate. The absorbance was measured every 24 h after agitation to re-suspend the algae. They were stored at 20 °C under cool white fluorescent lamps in a random order. The data was statistically analyzed using the Algaltox kit F Data treatment ErC50 spreadsheet.

2.6.3. Tetrahymena thermophila toxicity tests

The 24 h protozoan growth inhibition bioassay was performed using Protoxkit F (Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium). *Tetrahymena thermophila* were chosen for toxicity testing due to their sensitivity to a variety of emerging organic contaminants at environmentally relevant concentrations, as well as their position within the ecosystem and the potential for further bioaccumulation [16].

The test was carried out in disposable spectrophotometric cells of 1 cm path-length to measure changes in optical density (OD) at 440 nm. Each test cell contains *T. thermophila* ciliate inoculum (40 µL), food substrate (40 µL) and known concentration of the isomer or isomers being tested in synthetic freshwater (pH 7.75 ± 0.19, 2 mL). The OD measurements were taken at T0 h and at T24 h. At T0 h the turbidity of the test cell will be high due to the food substrate. Over 24 h the turbidity will drastically decrease as the uninhibited growth of the ciliates will consume the food substrate. The degree of inhibition can be calculated from the difference in OD between the control cells and the test cells after 24 h.

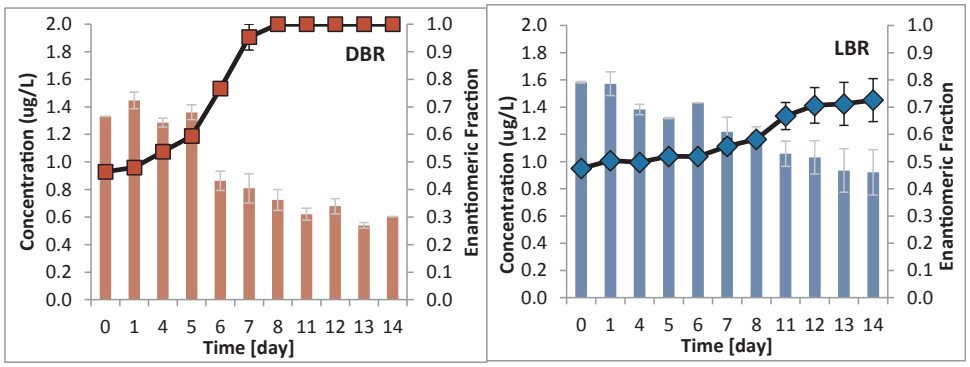
A preliminary investigation was carried out for 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine to ascertain the approximate range between 100% inhibition and uninhibited growth across 4 orders of magnitude. Based on these results (see Supplementary Tables 14 and 17), further definitive tests were carried out for each isomer between the lowest concentrations with a population growth inhibition of 80–100% and the highest concentration with an inhibition between 0–20%. To ensure the tests were valid each concentration was examined in duplicate and the control had to reach 60% OD decrease after 24 h. Some tests required an extra 2–4 h to reach the validation criteria, which was batch dependent and indicated a slightly slower growth of the ciliates. The EC50 values for this study were calculated using 28 h results.

3. Results and discussion

3.1. Stereoselective degradation of a mixture of ephedrine stereoisomers in river simulating microcosms

Degradation of a mixture of all four ephedrine stereoisomers and norephedrine enantiomers was studied in well-defined laboratory river water microcosm experiments. The following parameters were investigated: microbial degradation, photochemical reactions as well as other physicochemical processes such as sorption.

As can be observed from Fig. 1 during a 14-day period (±)-ephedrine degradation only occurs in biotic microcosms, which indicates that microbial metabolic processes are the main degradation pathway for this compound in the environment (under studied experimental conditions). It is worth noting that the rate of biodegradation is higher in dark biotic microcosms than in those exposed to light. This shows that ephedrine is photostable under the experimental conditions. Furthermore, biotic degradation of ephedrine shows high stereoselectivity favouring degradation of natural 1R,2S-(-)-ephedrine and leading to enrichment of (±)-ephedrine with synthetic 1S,2R-(+)-enantiomer. This process is much more pronounced in dark biotic microcosms.



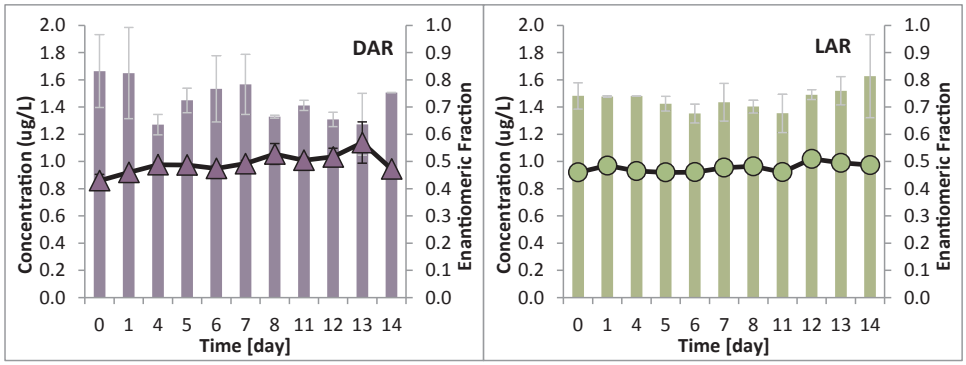
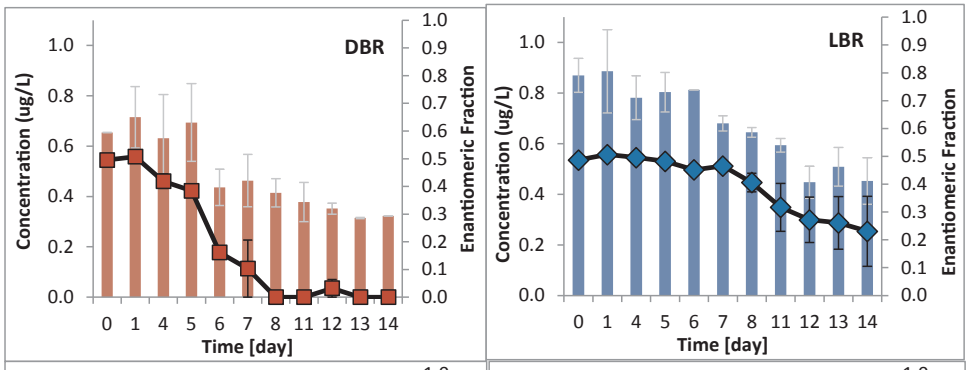


Fig. 1. Mixed-compound river simulating microcosms – (±)-ephedrine degradation under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols). See Tables S10-S13 for raw data.



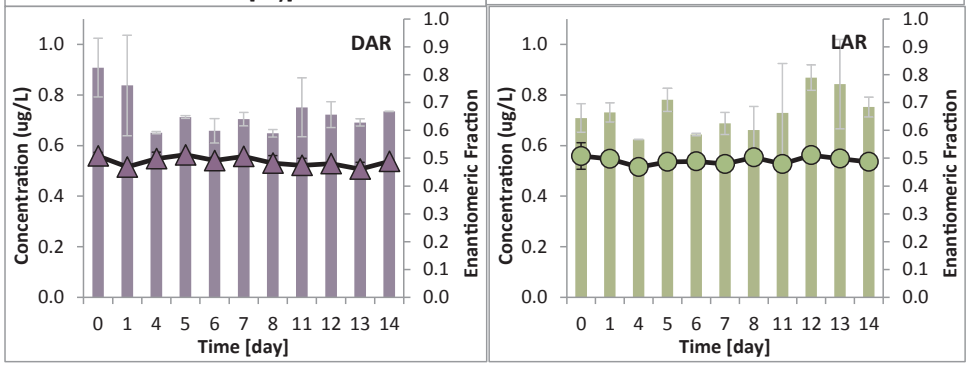


Fig. 2. Mixed-compound river simulating microcosms – pseudoephedrine degradation under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols). See Tables S10–S13 for raw data.

Pseudoephedrine was found to degrade in a similar manner to ephedrine (Fig. 2). It shows high photostability in both biotic and abiotic microcosms and no changes in enantiomeric composition were observed in both light and dark abiotic microcosms, whilst microbial metabolic processes are effective in the degradation of pseudoephedrine. These processes show high stereoselectivity with preferential degradation of 1S,2S-(+)-pseudoephedrine and subsequent enrichment of pseudoephedrine with synthetic 1R,2R-(-)-enantiomer. Similarly to ephedrine, degradation of pseudoephedrine is faster and shows higher stereoselectivity in the absence of external light. The increased rate of 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine degradation in the biotic dark microcosms compared to biotic light might be due to the growth of algae in the presence of light and needs to be investigated further.

Similar to ephedrine and pseudoephedrine, degradation of norephedrine was observed in the biotic microcosms only indicating an importance of microbial metabolic processes (Fig. 3). (±)-Norephedrine was examined in this experiment due to its potential importance as a breakdown product of (±)-ephedrine in animals and man [5–8], however significant (±)-norephedrine formation has not been

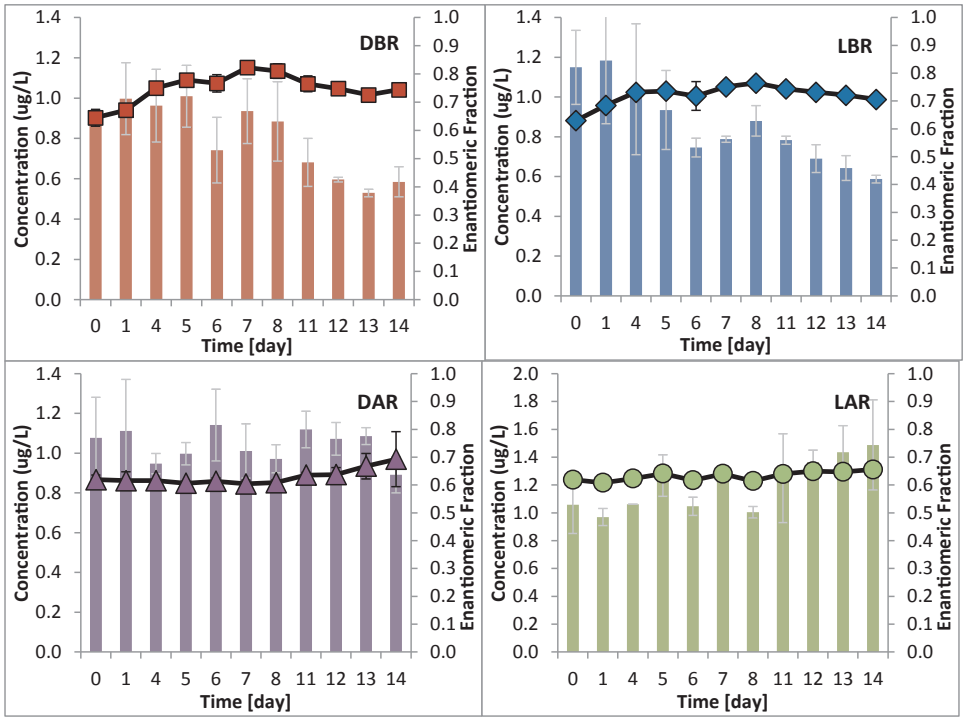


Fig. 3. Mixed-compound river simulating microcosms – norephedrine degradation under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols). See Tables S10–S13 for raw data.

observed in either of the biotic microcosms. Daily pH, temperature and dissolved oxygen (DO) measurements on each microcosms showed little variation between replicate samples (Fig. S2), as well as limited inter-microcosm variation.

3.2. Single 1R,2S-(-)-ephedrine or 1S,2S-(+)-pseudoephedrine river simulating microcosms

In the light of the observed extensive biodegradation of naturally occurring 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine, single isomer 1R,2S-(-)-ephedrine or 1S,2S-(+)-pseudoephedrine river simulating microcosms were undertaken to verify if chiral inversion leading to the formation of non-natural 1S,2R-(+)-ephedrine or 1R,2R-(-)-pseudoephedrine can occur in the aqueous environment (Fig. S3). Formation of 1S,2R-(+)-ephedrine was observed in a single-isomer biotic-light 1R,2S-(-)-ephedrine microcosm (Fig. S5). It is important to note that the river water used in the microcosm experiments did not contain any ephedrine isomers prior to the start of the experiment. The observed peak passed method identification criteria, including retention time and MRM transition ratios, and is possibly formed as a result of chiral inversion, albeit after long residence time of 14 days and despite limited degradation of 1R,2S-(-)-ephedrine (Fig. S6). This is a very important result, as it matches the observation of Kasprzyk-Hordern and Baker [1] showing formation of 1S,2R-(+)-ephedrine after biological wastewater treatment. The limited degradation of 1R,2S-(-)-ephedrine in this experiment (explained by matrix collection during heavy rainfall and low biomass content) has hindered the observation of metabolite formation within early stages of the experiment.

3.3. Human liver microsome assays to verify human metabolism of 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine

Human liver microsomes assays were utilized to verify if human metabolism can contribute to environmental occurrences of 1S,2R-(+)-ephedrine via stereoselective metabolic processes of ephedrine isomers including chiral inversion. Across the six hours of incubation a sample was taken and quenched at 1, 3 and 6 h after addition of HLMs. At each of these time points there was no significant change in the concentration of 1R,2S-(-)-ephedrine relative to the control samples (Table S5), this is in line with literature data showing that 1R,2S-(-)-ephedrine is generally excreted unchanged in humans [5,6]. This is further supported by the absence of (±)-norephedrine or any other ephedrine isomer, which were expected to be the main metabolites [5,6] if 1R,2S-(-)-ephedrine was metabolised. The concentration of 1S,2S-(+)-pseudoephedrine however did decrease relative to the control sample, but no (±)-norephedrine or any other ephedrine isomers were observed. The HLM experiments for both compounds therefore support the available literature data that ephedrines are mainly excreted un-metabolised and without chiral conversion. This also eliminates human metabolism of ephedrines as a source for the previous detection of 1S,2R-(+)-ephedrine in wastewater [1].

3.4. Retrospective analysis for 1S,2R-(+)-ephedrine precursors in river water

It is important to mention that there could be other sources of 1S,2R-(+)-ephedrine formation in water such as the reduction of R-(+)-methcathinone during human metabolism [17]. (±)-Methcathinone has been occasionally detected in wastewater influent [18,19] but not in other environmental samples, and never with a focus on chirality. However as no ephedrine isomers were present in the river water used for microcosm experiments, and as relatively low percentages of methcathinone are excreted un-metabolised [17], it is postulated that 1S,2R-(+)-ephedrine was formed as a result of microbial transformation of 1R,2S-(-)-ephedrine. Further work is needed to confirm this hypothesis, however retrospective analysis of river water collected in a week long sampling campaign in England, using the procedure detailed in [13], showed no evidence of methcathinone.

3.5. Ecotoxicity of ephedrine stereoisomers to Daphnia magna, Pseudokirchneriella subcapitata and Tetrahymena thermophila

Ecotoxicity tests and obtained EC50 data for the non-natural isomers *1R,2R*-(-)-pseudoephedrine and *1S,2R*-(+)-ephedrine revealed that these isomers are more toxic to *D. magna* than their naturally occurring enantiomers (Table 2). Their EC50s after 48 h exposure were as

Table 2
Toxicity of ephedrine stereoisomers to *Daphnia magna, Pseudokirchneriella subcapitata* and *Tetrahymena thermophila*. Raw data is presented in Figs S7–S11, Tables S6–S9 and Tables S14–S21.

Single isomers	*D. magna*		*P. subcapitata*	*T. thermophila*
	$EC50_{24h}$ [mg/L]	$EC50_{48h}$ [mg/L]	$EC50_{72h}$ [mg/L]	$EC50_{24h}$ [mg/L]
1S,2R-(+)-Ephedrine	373.1	170.8	754.5	42.6
1R,2S-(-)-Ephedrine	408.6	253.7	259.1	36.0
1S,2S-(+)-Pseudophedrine	528.3	274.3	417.9	99.3
1R,2R-(-)-Pseudophedrine	128.2	107.2	44.8	4.6
Mixtures				
Natural	–	–	–	61.8
All	–	–	–	52.4

follows: 107 and 171 mg/L in the case of *1R,2R*-(-)-pseudoephedrine and *1S,2R*-(+)-ephedrine respectively. $EC50_{48h}$ for the natural isomers *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine, being widely used as prescription and over-the-counter medications, were much higher: 253 and 274 g/L respectively.

Similarly, non-natural *1R,2R*-(-)-pseudoephedrine was found to be the most toxic isomer (< 100 mg/L) for *P. subcapitata* after 72 h exposure time. Interestingly, *1S,2R*-(+)-ephedrine was found to be less toxic than its natural isomer. *1R,2S*-(-)-ephedrine and was found to be the least toxic isomer.

The results show *T. thermophila* are far more sensitive to the presence of the ephedrine isomers than the other organisms explored in this study. As with the other organisms *1R,2R*-(-)-pseudoephedrine is by far the most toxic and its enantiomer *1S,2S*-(+)-pseudoephedrine the least: 4.6 and 99.3 mg/L, respectively. The ephedrine enantiomers *1S,2R*-(+)-ephedrine and *1R,2S*-(-)-ephedrine are much more similar in toxicity: 42.6 and 36.0 mg/L, respectively. As with *P. subcapitata* 1R,2S-(-)-ephedrine was more toxic than 1S,2R-(+)-ephedrine, although with less disparity.

The *T. thermophila* $EC50_{24h}$ results for the ephedrine mixtures 'natural' (containing *1S,2S*-(+)-pseudoephedrine and *1R,2S*-(-)-ephedrine, DF = 0.5) and 'all' (which contains all the isomers, all EF and DF = 0.5) are 61.8 and 52.4 mg/L respectively. These two mixtures are less toxic than all of the individual isomers apart from *1S,2S*-(+)-pseudoephedrine, which is present in both mixtures, so it does not appear that there are any synergistic effects. All *T. thermophila* $EC50_{24h}$ results are classified as harmful (< 100 mg/L), however *1R,2R*-(-)-pseudoephedrine is toxic (1–10 mg/L) according to the classification made by OECD and Commission of the European Communities [20–23]. For more detailed results please see Supplementary Tables 14–21.

4. Conclusions

This manuscript examined the degradation of each of the four isomers of ephedrine in river simulating microcosms and verified toxicity of each isomer to aquatic test organisms (*D. magna, P. subcapitata and T. thermophila*). Microcosms showed that significant degradation only occurred in biotic conditions and that only two isomers (*1R,2S*-(-)-ephedrine, *1S,2S*-(+)-pseudoephedrine) degraded significantly over a period of fourteen days. Across all three organisms tested the persistent isomers (*1S,2R*-(+)-ephedrine and *1R,2R*-(-)-pseudoephedrine) were more toxic than those that undergo degradation.

The high toxicity of *1S,2R*-(+)-ephedrine is of significant environmental importance. Kasprzyk-Hordern and Baker [1] have detected *1S,2R*-(+)-ephedrine in wastewater effluent, assumed to be formed during wastewater treatment, which is eventually discharged into the environment. HLM assays support previous literature evidence that human metabolism of ephedrines does not proceed with any conversion of chirality, so that 1S,2R-(+)-ephedrine formation is occurring after excretion or during wastewater treatment. Multi-compound microcosm experiments show that, if present, *1S,2R*-(+)-ephedrine is persistent within the environment, allowing it to become more widely spread. The detection of 1S,2R-(+)-ephedrine in single isomer microcosms containing only

1R,2S-(-)-ephedrine suggests that chiral inversion from biological processes is possible, further increasing the risk of pseudo-persistent environmental exposure to 1S,2R-(+)-ephedrine and that chiral inversion could occur during biological treatment of wastewater containing 1R,2S-(-)-ephedrine.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.01.020.

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Chapter 6

Conclusions

1.1 Development and validation of a multi-residue method for exposure-driven catchment-based study

This thesis initially identified the current limitations of existing exposure driven studies, such as lack of harmonised methods, lack of monitoring solid phases and limited range of investigated CECs. These limitations reduce the ability to compare between studies, and the environmental burden is often underestimated. Therefore, a list of 56 CECs covering 20 classes, was prioritised by reviewing existing regulation, literature, physicochemical parameters, persistence, metabolism and excretion. These were combined with 90 additional CECs covering 26 classes from another method (Petrie et al., 2016), and validated for quantitative analysis in 5 matrices, 3 liquid and 2 solid, across a catchment. The resultant validated method included a range of 142 analytes covering 34 classes. This is one of the largest multi-residue methods published, which can quantify CECs in both liquid and solids matrices relevant to WwTW processes and environmental exposure to concentrations below current PNECs (Proctor et al., 2019).

1.2 Application of method to multiple matrices to evaluate the impact of WwTWs on receiving waters and micropollutant fluxes

This method was applied in a catchment-based study in 2015 to quantify CECs across a catchment in the South-West of the UK, at 5 WwTWs covering >75% of the ~1.5 million population. Each WwTW had samples collected from influent, effluent, upstream and downstream for 7 consecutive days. SPM was filtered from each influent sample for solids preparation and analysis, and digested solids were collected from sites with sludge treatment facilities. This comprehensive high-resolution work found 112 out of 138 CECs in liquid influent, 76 out of 98 in SPM, 106 out of 137 in effluent, 50 out of 138 upstream of the first WwTW studied, 84 out of 138 downstream and 65 out of 96 in digested solids (Proctor et al., 2020).

Analysis of influent and SPM allowed the partitioning of solids to be quantified prior to coagulant addition and primary treatment. This is key for wastewater-based epidemiology (WBE) approaches and an accurate understanding of temporal and spatial trends in the usage and disposal of these CECs. Several spatial and temporal trends were identified between urban and rural areas, and between the working week and weekends. A potential incident of direct disposal of carbamazepine was also identified.

Population, urbanisation and industry are key to the CEC levels found in influent. SPM was found to be the primary matrix for several classes and certain compounds. This finding shows that only analysing the liquid phase of influent in previous studies will have led to an underestimation of those CECs. WwTWs showed effective removal from the liquid phase for many classes, however many of these classes are of particularly high usage and enter the WwTW at a very high level, therefore despite high removal they are still present in effluent and discharge to the environment at a significant level.

Many CECs are found throughout the aquatic environment, largely introduced from the effluent of WwTWs at a continuous, low, anticipated level. Some are present due to unexpected or accidental events, such as direct disposal, which may cause short bursts of high concentrations, often leading to higher solid partitioning of the CEC within influent. Whilst the acquisition of downstream/upstream samples in the aquatic environment were subject to timing limitations, of the samples taken, some CECs showed no degradation between sites. Measurements taken down the river showed an increasing trend in overall CEC load and concentration. This is concerning, as it indicates the environment is overburdened and cannot degrade the CECs at the same rate they are entering, leading to pseudo-persistent CECs being transported throughout the aquatic environment that may reach more sensitive areas of the catchment. Based on these findings, a catchment-based approach to risk assessment should be an important consideration for the environmental impact of new chemicals and medicinal products in future.

The aquatic organisms are not the only organisms at risk; this work showed potential exposure to terrestrial organisms from digested solids, if applied to the terrestrial environment. A wide range of CECs have been found in digested sludge, including many which were previously not considered or expected to be present in solids. This is likely due to very high concentrations entering the WwTW, leading to detectable concentrations in the solid phase despite poor partitioning. This may be followed by a lack of removal in solid treatment processes. If then applied to soil, this may result in the presence of weakly bound CECs entering the environment, where they have the mobility to be transported to groundwater, surface waters or crops.

1.3 Assessment of environmental risk of CECs from a catchment perspective

This work also considered the potential effects of these levels of exposure. Using acute and chronic aquatic ecotoxicity data from literature and quantitative structure activity relationships (QSAR) modelled data to devise appropriate PNECs for all CECs, it was found several classes posed an acute risk to the environment (based on $RQ_{MEC:PNEC}$ >1, antibiotics ($RQ_{MEC:PNEC}$ up to 33.29), NSAIDs

($RQ_{MEC:PNEC}$ up to 1.71), lipid regulators ($RQ_{MEC:PNEC}$ up to 2.98), lifestyle chemicals ($RQ_{MEC:PNEC}$ up to 78.88), and veterinary pharma ($RQ_{MEC:PNEC}$ up to 31.68). Overall, lifestyle chemicals showed the highest acute risk to the environment. The chronic PNECs indicated other classes may also be of risk, including; plasticiser ($RQ_{MEC:PNEC}$ up to 206.86), antifungals ($RQ_{MEC:PNEC}$ up to 3.51), antidiabetics ($RQ_{MEC:PNEC}$ up to 2.60), antidepressants ($RQ_{MEC:PNEC}$ up to 1.55), and pesticides ($RQ_{MEC:PNEC}$ up to 5.39). As well as these chronic assessments, several classes were identified to be of acute risk, such as lifestyle chemicals, which also showed a chronic $RQ_{MEC:PNEC}$ of 283.11. Overall this assessment showed that lifestyle chemicals and the plasticiser, bisphenol A, pose the most long-term risk to the environment.

The overall mixture at each river sampling site was also assessed, showing an overall chronic $RQ_{MEC:PNEC}$ of up to 516.5 and an acute $RQ_{MEC:PNEC}$ of up to 105. This shows that these mixtures are likely to pose a risk to the environment throughout the catchment. However, this is for a mixture of dissimilarly acting chemicals and in such cases, RQ_{STU} has been suggested as a more appropriate criterion, as it assesses the risk of the mixture to each trophic level individually before calculating the RQ_{STU} for the trophic level most at risk. The acute RQ_{STU} shows the algal trophic level was most at risk, with an RQ_{STU} up to 93.4 and the most significant contributor was veterinary pharma, specifically diazinon. The chronic RQ_{STU} showed the daphnid trophic level was most at risk, with RQ_{STU} up to 425.2, the most significant contributor was the plasticiser, bisphenol A. This shows a handful of CECs may pose the greatest environmental risk, as previously discussed by Backhaus and Karlsson, as well as others, therefore it is very important to ensure a wide range of CECs are investigated (Backhaus and Karlsson, 2014; Gustavsson et al., 2017; Verro et al., 2009). If key CECs are not included in the investigation, it may lead to an underestimation of environmental risk in the subsequent assessment.

Unsurprisingly, spatial ecotoxicity trends showed an increasing environmental risk downstream. The chronic RQ_{STU} and $RQ_{MEC:PNEC}$ increased downstream across the catchment by 35 times and 5.2 times respectively. The acute RQ_{STU} and $RQ_{MEC:PNEC}$ increased by 3.8 and 3.3 times respectively. Furthermore, there was a high risk to the environment before the most upstream sampling point, suggesting that other smaller WwTWs still pose a significant risk to the environment, as well as other more diffuse sources.

The analysis of the risk to the terrestrial environment was more difficult due to the lack of available ecotoxicity data in literature and modelled QSAR data. The environmental risk was considered for both predicted environmental levels in soil after application of digested solids and for undiluted digested solids, to estimate localised risk posed by inadequate mixing. Most available data focussed only on earthworms. Therefore, assessments for both the whole mixture and individual classes could not be completed, due to lack of information for all compounds detected.

The same ecotoxicity data was applied to both amended soils and undiluted digested solids. For amended soils, the acute ecotoxicity data showed there was minimal risk to terrestrial organisms, as the most toxic CEC was gemfibrozil, with a negligible $RQ_{PECsoil:PNEC}$ of 0.01. The chronic ecotoxicity, for the three CECs for which it could be found; ibuprofen, methylparaben and bisphenol A, showed no environmental risk. For undiluted digested solids however, the acute ecotoxicity data showed high risk to the environment for individual CECs. The highest $RQ_{MEC:PNEC}$ was from gemfibrozil was 7.03. Ketoconazole and diclofenac also posed a significant acute risk at the levels detected, with $RQ_{MEC:PNEC}$ up to 1.91 and 1.07 respectively. The chronic test data for ibuprofen, methylparaben and bisphenol A, showed medium to high risk to the terrestrial environment with $RQ_{MEC:PNEC}$ of up to 0.43, 1.54 and 10.8 respectively. Even with this limited ecotoxicological dataset, undiluted, this matrix shows high risk to the environment.

Overall, the utilised quantitative method provided MQLs lower than the PNEC for most compounds in the aquatic environment, showing this method is suitable for the environmental risk assessment (ERA) for this mixture of CECs. However, conclusions could not be drawn for the suitability of the method for assessing environment risk of CECs to the terrestrial environment, due to lack of sufficient ecotoxicological data.

1.4 Development of novel approaches and further ecotoxicological considerations

Several aspects were not considered during this ERA, including metabolites, transformation products and the effect of stereochemistry. Though several metabolites were included in this study, there was limited ecotoxicity data available for the ERA. Guidelines on the ERA of medicinal products suggest ERA should only be carried out on metabolites which are excreted > 10% of the administered dose. Therefore, metabolites such as norfluoxetine would not be considered for ERA. However, based on the findings of this work, such as the toxicity the enantiomers of fluoxetine compared to its metabolite norfluoxetine, it is advised that at least a preliminary assessment for all metabolites is undertaken. This is because it has been shown that the metabolite can be significantly more toxic than the parent or may have the potential for synergistic effects to compound toxicity; if the metabolite is ignored just because it is excreted at a low rate, the subsequent ERA may underestimate the overall environmental risk. Furthermore, this work provided evidence that these metabolites are also produced during wastewater treatment.

Most of the available literature on environmental monitoring of CECs focuses on the achiral analysis. This work briefly explores the stereochemistry for a few chiral CECs in this method. It finds that enantiomeric fractions (EFs) vary between different matrices and are not present as a racemic mixture.

This can have important ramifications on the ecotoxicity and existing ERAs, which are currently based on the initial formulation produced, or in the case of pharmaceuticals, based on the predominant metabolised form. Therefore, the EFs present in the environment may cause different biological effects. This is an under investigated aspect of ecotoxicological work. The effect of stereochemistry on ecotoxicity was explored in depth for ephedrine and fluoxetine and showed that the ecotoxicological impact for different stereoisomers can be drastically different, such as for one fluoxetine enantiomer, which is 35 more toxic for *T. thermophila* than the other enantiomer. Furthermore, different organisms may have a different toxic response to a range of EFs, therefore a wide range of test organisms need to be investigated. Overall, more ecotoxicity data on chiral compounds would increase the accuracy of ERAs but the study of which is currently limited by the availability of enantiomers in a suitable and affordable form.

This work also explored the development of a 'nanotest' for the *T. thermophila* which allowed a far lower level of toxicant to be tested at relevant concentrations, allowing a full dose-response curve to be developed for this organism, which can be found in a range of environment matrices.

Overall, the CEC loads seen in the aquatic environment and digested solids are likely to increase if nothing is done to mitigate CEC usage and/or release, making this an issue of critical importance. With the increasing and aging population in the UK, and continued urbanisation, catchments like the one studied in this work are at high risk of increasing levels of exposure to CECs in the environment. CECs with low removal levels by WwTW processes, or CECs which are poorly metabolised, are a particular risk of increasing presence in environmental matrices. Furthermore, consideration of a single compound as a mixture of its metabolites, transformation products or even as a different ratio of its enantiomers (if chiral), is of upmost importance to fully understand the risk a compound may pose to the environment.

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Chapter 7

Further work

1.1 Future developments in multi-residue methods for exposure-driven approaches

Though the multi-residue method developed and validated in this work covers one of the widest range of CECs using a single preparation method, it still only covers a fraction of the potential CECs in the environment. Review of recent literature shows work is being carried out in developing screening methods using high-resolution mass spectrometry to scan far a larger range of CECs against libraries (Pesticides HRMS ref) and up until recently, sensitivity, was a major limitation of this methods, with their inability to detect the lower PNEC ranges in the environment. However, with recent advances in high-resolution mass spectrometry soon this may not be a problem.

A key aspect of further work is the inclusion of metabolites and transformation products in future sampling campaigns and ecotoxicity work, currently available data on both aspects is very limited and therefore the true environmental risk of these compounds is unknown. Though it is often assumed to be negligible, there is not enough data for this assertion to be valid. However, further work considering non-targeted analysis with high-resolution mass spectrometry will be critical in the identification of metabolites and transformations products from WwTW and determining their impact.

1.2 Further considerations for future catchment studies

This was a comprehensive catchment-based study and essential data was gathered on a number of critical points discussed herein. However, these studies are limited by currently available sampling and sensing technology. With better tools this may allow spatial and temporal variation in concentration of CECs to be tracked with more reliability and provide a more realistic assessment for chronic levels.

The catchment study has shown the potential for several compounds to absorb to sediments, particularly antidepressants and diclofenac. Both these compounds should be investigated further, as well as others which have shown an affinity for partitioning to the solid phase. As there are many benthic organisms at risk from these concentrations within the sediment, it should also be assessed for environmental impact. Furthermore, the spatial trend of increasing CEC concentration downriver is likely to be mirrored in the sediment, therefore multiple locations throughout the catchment should be observed.

The estuarine environment is potentially at high risk due to the increasing CEC levels downstream towards the estuary, but also from close proximity to the output of the largest WwTW in the catchment, which receives influent from the greatest population equivalent. Though the mass balance showed this WwTW had the best removal per person overall, due to the quantity received, it also had the largest output. The potentially high levels of CECs leaving the catchment and entering this area suggest future work should strongly consider including estuary sampling in future catchment monitoring. Previous studies in Sweden have shown that CECs are present at detectable levels within the marine environment (B. M. Gustavsson et al., 2017) and this is compounded by their potential to bioaccumulate within marine organism (Mezzelani et al., 2018), which shows that if the estuary is becoming contaminated, it is an important issue.

1.3 Future perspectives on environmental risk assessment of CECs

Overall, with regards to environmental risk assessment, there is a need for more publicly available ecotoxicity data with more detailed accounts of research methods, to encourage harmonisation of future methods, and to produce more consistent PNECs and ERAs. Moreover, the collection of data within databases such as ECOTOX and TOXNET, as well as the availability of modelled data from sources such as ECOSAR, can provide a further level of consistency across environmental risk assessments in literature.

Chronic ecotoxicological data is seen as far more applicable to the environment as there is a constant anticipated release into surface water or long-term presence within the terrestrial environment and yet this data is still far less available than acute data. Furthermore, the lower effect concentration levels, such as NOEC and LOEC are not always statistically defined, as they are just based on the lowest concentration tested and therefore the environmental risk assessment using this may be misleading.
The presence of CECs in digested solids and the lack of relevant ecotoxicity data, indicates a key area of further work, for both individual CECs and mixtures. Furthermore, the removal of CECs from solids during treatment to digested solids is unknown, which warrants further investigation. This is particularly important due to the potential hazard of these CECs in the environment after application of digested solids to soils.

The risk assessment of mixtures carried out in this study, as well as other studies, has shown that a few constituent compounds may account for most of the toxic effect (Backhaus and Karlsson, 2014; M. Gustavsson et al., 2017; Verro et al., 2009). However, different studies have shown these key CECs to differ; this may be due to a limited selection of investigated CECs in each study, further emphasising the need to quantify as many CECs in the environment as possible.

1.4 Future development and ecotoxicological considerations

Bioaccumulation, bioconcentration and other sublethal effects were not considered in this work. However, this is still critical information for assessing the wider impact of the CECs in the environment. Sublethal effects and behavioural studies show responses at levels several magnitudes lower than the standard ecotoxicity endpoints. However, these methods still require further work to improve reproducibility and understanding of the potential ecological implications, as well as optimisation and harmonisation of methods before they can be used more widely (Kohler et al., 2018).

Seasonal trends should be a further consideration for future catchment-based sampling campaigns, particularly in consideration of the impact that seasonal variations of the CEC content of mixtures may have on the lifecycle of flora and fauna throughout the year. As previous studies have shown, there can be seasonal trends in CEC removal, pharmaceutical usage and environmental concentrations (Burns et al., 2018; Golovko et al., 2014; Ma et al., 2017; Papageorgiou et al., 2016; Tsui et al., 2014).

Overall, approaches which combine the use of exposure and effects-based methods are needed. High resolution methods which can simultaneously analyse the chemical exposure and biological effect would be ideal and are in fact applied in the field of environmental metabolomics. This approach uses methods to analyse the exogenous and endogenous metabolites that an organism(s) produces in response to a stressor. This stressor may be physical, such as temperature or lack of food, or it may be chemical e.g. exposure to a CEC. Depending on the stressor, changes will occur in the organism's metabolome. The potential for this approach is high, but an understanding of which stressors causes what effects is still required for the biological interpretation of the data collected (Lankadurai et al., 2013; Miller et al., 2018).

Understanding the levels of exposure and their effect is of high importance. However, research has shown that there is currently a high level of risk to the environment and with increasing population and levels of urbanisation, this is only going to get worse. Therefore, further work should also be undertaken to take mitigative steps.

Firstly, better optimisation of current removal processes could be achieved, as many of the processes are not designed for the removal of CECs. The evidence shows that current technology could be improved or expanded e.g. improving CEC sorption to solids, increasing retention time and introduction of tertiary treatments such as membrane bioreactors (MBRs) (Grandclément et al., 2017) and reed beds (Gardner et al., 2013; Petrie et al., 2018).

Secondly, pre-treatment of highly potent CEC sources e.g. hospitals and trade-effluents, may reduce the burden on WwTWs. This is currently implemented for common aspects such as sanitary determinands and pH but is yet to be widely implemented with regards to CECs.

Thirdly, source control should be considered. This includes spreading public awareness including the overall picture of CECs in the environment, correct disposal practices of CECS, and reducing unnecessary usage e.g. minimising unnecessary pharmaceutical use. Other than public awareness it could include banning particularly toxic and persistent CECs, such as pesticides, and development of 'greener' alternatives to CECs which are designed to degrade and be less persist and toxic (Taylor and Senac, 2014). Further changes may also be made to the guidelines for regulation of CECs, their registration into the open market and continued use. Currently these guidelines do not consider mixtures of similarly acting compounds, stereochemistry of CECs and their enantioselective transformation. Consideration for impact of CECs on the terrestrial environment is low and could be improved further, though recent changes to the 'Guidelines for ERA of medicinal products' now considers soils to be potentially at risk from pharmaceuticals with K_{oc} <10,000 L kg^{-1} in sludge and considers a range of K_{oc} for sludge which trigger further ERA and PEC of surface water (European Medicines Agency (EMEA), 2006; European Medicines Agency, 2018; Whomsley et al., 2019). This is likely to improve the assessment of the terrestrial environment for compounds like ibuprofen.

Finally, efforts towards bioremediation should be made. Current research shows many CECs and priority substances are highly persistent in the environment. For example, legacy pesticides, which have been banned in the EU for many years, are still appearing in storm water in catchments with large areas of agriculture, due to their persistence within agricultural soils. Furthermore, their preferences to partition to solids rather than liquids may pose a particular environmental risk to benthic organisms once they enter surface waters (Rasmussen et al., 2015). For these highly persistent compounds, bioremediation efforts could help accelerate removal and degradation of them, mitigating the chance that they will be transported to more fragile ecosystems where they may cause more damage.

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Outputs

Publications

Published

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De Groof V, Arnot T, Kasprzyk-Hordern B, Proctor K, Lanham A, Coma M. Emerging contaminants in food waste digestion. (In preparation)

Presentations

Oral:

- 2018: *11th Network Conference on Persistent Organic Pollutants, University of Birmingham, UK*
    - Proctor K, Andrés-Costa MJ, Barden R, Kasprzyk-Hordern B. The effect of stereoselectivity on the life-cycle, fate and effects of priority organic pollutants.

- 2017: *Industry presentation with Wessex Water and Astra Zeneca, University of Bath*
    - Proctor K, Kasprzyk-Hordern B. Enantiomer specific toxicity of fluoxetine and ephedrine

- 2015: *NERC Research Showcase: Associate Partners Event at Bath*
    - Proctor K, Barden R, Kasprzyk-Hordern B. Lifecycle of Emerging Contaminants in Urban Water: Novel (Bio)Analytical Approaches to Environmental Risk Assessment.

Poster:

- 2020: *SETAC SciCon: SETAC Europe 30th Annual Meeting*
    - Proctor K, Petrie B, Lopardo L, Camacho-Muñoz D, Rice J, Barden R, Arnot T and Kasprzyk-Hordern B. CECs in the Urban Environment: A Catchment Perspective

- 2018: *Emerging Analytical Professionals 'The Circle of Life – Analysing the World Around Us'*
    - Proctor K, Andrés-Costa MJ, Barden R, Kasprzyk-Hordern B. The importance of stereoselectivity in understanding the life cycle, fate and effects of emerging contaminants.

- 2017: *18th UK Young Water Professionals Conference 2017: A Water World Without Boundaries*
    - Proctor K, Petrie B, Youdan J, Barden R, Arnot T, Kasprzyk-Hordern B. Lifecycle of Emerging Contaminants in Urban Water: Novel (Bio)Analytical Approaches to Environmental Risk Assessment.

- 2016: *Analytical Science Network's Bright Spark Symposium 2016*
    - Proctor K, Petrie B, Youdan J, Barden R, Arnot T, Kasprzyk-Hordern B. Lifecycle of Emerging Contaminants in Urban Water: Novel (Bio)Analytical Approaches to Environmental Risk Assessment.

Secondments

- 2019**:** ***Wessex Water – (6 weeks) -*** *GW4 FRESH NERC CDT NPIF Innovation Placement, Bath, UK*
- 2018: ***INTERWASTE – (2 months) -*** *Synergising International Research Studies into the Environmental Fate and Behaviour of Toxic Organic Chemicals in the Waste Stream, Mendoza, Argentina*
- 2017: ***Wessex Water – (3 months) -*** *Development and commission of a sampling strategy with for the Public Health Project, Bath, UK*

Outreach Activites

Public Engagement

- 2018**:** Participated in outreach event – science day Coombe Down Primary School
- 2017: Presented at 'Girls into Maths A Level' day, Maths Hub, University of Bath
- 2017: Participated in outreach event – science day Coombe Down Primary School